REMARKS/ARGUMENTS

Upon entry of the current amendment, claims 1-10, 13 and 16-22 are pending in the present application. Applicants have amended claims 1-10, 13, 16 and 21 and canceled claim 11. Support for the amendment to claim 1 can be found in the published application at paragraphs 0009, 0011-0012 and 0016, and in originally filed claim 11. Claims 2-10 and 13 are amended to address the Examiner's objection and for consistency with amended claim 1 from which they depend. Support for the amendments to claims 2-10 and 13 can be found in original claim 11. Support for the amendments to claims 16 and 21 can be found in the published application at paragraphs 0009, 0011-0012 and 0016.

The Examiner has rejected all of the pending claims, and applicants address each of the Examiner's comments in the order made.

1. Claim Objections

Applicants have amended claims 2-10 to replace "A" with "The" in accordance with the Examiner suggestion. *See* p. 2 of the OA. Withdrawal of the objection to claims 2-10 is respectfully requested.

2. Claim Rejections - 35 USC 112 Written Description

The Examiner has rejected claims 1-11 and 13 pursuant to 35 USC 112, first paragraph, as allegedly failing to comply with the written description requirement. The Examiner asserts that the claims contain subject matter not described in the specification in such a way as to reasonably convey to a skilled person that applicants had possession of the claimed invention at the time the application was filed. *See* p. 2 of the OA. In particular, the Examiner asserts that the specification discloses only recombinant human C1 inhibitor and the use of O-linked carbohydrate modifying enzymes ST3Gal I and ST3Gal III. *See* p. 3 of the OA. Thus, the Examiner concludes, the specification fails to disclose a sufficient number of examples to reasonably convey to the skilled person that applicants were in possession of the complete genera recited in the claims. *Id.*

Without agreeing with the Examiner's assertions, applicants have amended claim 1 to recite a recombinant *human* C1 inhibitor. The Examiner acknowledges that the specification

discloses recombinant human C1 inhibitors that have been modified with exemplary enzymes ST3Gal I and ST3Gal III. *Id.* Thus, the recitation of a recombinant human C1 inhibitor addresses the Examiner rejection with regard to C1 inhibitors.

Turning to the Examiner's assertions regarding the description of O-linked carbohydrate modifying enzymes, applicants submit that an adequate description is provided in the application in light of an extensive description of such enzymes in the prior art literature. The description of a class of O-linked modifying enzymes, including sialyltransferases, galactosidases, and endo-acetylgalactosaminidases (*see* p. 3, lines 1-5 of the application), and the representative examples in the specification are sufficient to comply with the written description requirement when coupled with the extensive descriptions of such enzymes in the literature as of the priority date of the application.

Harduin-Lepers *et al.* (*Glycobiology* 5(8):741-758 (1995)) reports 19 sialyltransferases, including ST3Gal I and ST3Gal III, as exemplified in the present application. *See* Table 1 of the Harduin-Lepers - Glycobiology reference. The same author presents an overview of 15 cloned and characterized human sialyltransferase enzymes in *Biochimie* 83:727-737 (2001). In addition, Cabezas *et al.* (*Int. J. Biochem.* 15(3):243-259 (1983)) reviews a plurality of glycosidases, including fucosidases, galactosidases, glucosidases, and hexosaminidases. Copies of the references are enclosed for the Examiner's review. Because the skilled person was in possession of this extensive literature regarding the O-linked carbohydrate modification enzymes, as described and exemplified in the application, applicants were also in possession of the described genus of O-linked carbohydrate modification enzymes as of the priority date of the application in compliance with the written description requirement.

Based on the foregoing, applicants respectfully request withdrawal of this ground of rejection.

3. Claim Rejections - 35 USC 112 Enablement

The Examiner has rejected claims 1-11, 13 and 16-22 pursuant to 35 USC 112, first paragraph, as allegedly not being enabled by the specification. The Examiner asserts that the specification, while being enabling for a C1 inhibitor whose circulatory half-life is changed by an *in vitro* O-linked carbohydrate modification, or for a method of such *in vitro* modification, does not enable any method for changing the circulatory half-life of a C1 inhibitor *in vivo*, as

broadly claimed. *See* p. 4 of the OA. The Examiner relies on the reasons of record set forth in the previous Office Action of June 1, 2007. *Id.*

In the Office Action of June 1, 2007, the Examiner asserted that because the specification provides working examples only of *in vitro* modification of the O-linked carbohydrates, one of skill in the art would not have been able to practice the invention using any method of *in vivo* modification of the glycoprotein without "undue experimentation." In the present Office Action, the Examiner asserts that applicants' previous arguments were found unpersuasive because the claims continue to encompass *in vivo* modification of O-linked carbohydrates on "any proteins in any non-human animal" by co-expression of the protein and the O-linked carbohydrate modifying enzymes. *See* paragraph bridging pp. 4-5 of the current OA.

Applicants have amended independent claim 1 to recite that the O-linked carbohydrate modification is carried out *in vitro* or "*in vivo* by co-expression of the recombinant human C1 inhibitor with one or more recombinant O-linked carbohydrate modifying enzymes in a cultured transgenic cell." Similarly, applicants have amended claim 16 to recite that the removal of the one or more non-sialylated O-linked carbohydrates from the glycoprotein is carried out *in vitro* or "*in vivo* by co-expression of a recombinant glycoprotein with one or more recombinant enzymes capable of removing the one or more non-sialylated O-linked carbohydrates of the recombinant glycoprotein in a cultured transgenic cell." Thus, the Examiner's concern regarding the unpredictability of O-linked carbohydrate modification in non-human transgenic animals is moot in light of the present claim amendments. Moreover, the claims, as amended, recite co-expression of a recombinant glycoprotein and a recombinant O-linked carbohydrate modification enzyme, and modification of the recombinant glycoprotein, thus addressing the Examiner's concern that the claims encompass the modification of O-linked carbohydrates on *any* protein.

In light of the claim amendments, applicants reiterate that a working example of a particular embodiment (*e.g.*, *in vivo* modification by co-expression of a recombinant glycoprotein and a recombinant O-linked carbohydrate modifying enzyme in a cultured transgenic cell) is not required for enablement. *See* MPEP 2164.02. In the present case, one skilled in the art as of the priority date of the application would have readily appreciated from the

teachings of the specification the correlation between the description of *in vitro* modification of the O-linked carbohydrate moieties on the recombinant human C1 inhibitor provided in the examples and the description of *in vivo* modification in cultured transgenic cells through co-expression of the recombinant glycoprotein (*e.g.*, a human C1 inhibitor) and one or more recombinant O-linked carbohydrate modifying enzymes, as described in the specification (*e.g.*, paragraphs 0011-0012 of the published application), and as presently claimed.

Applicants also reiterate that the skilled artisan could readily determine the extent to which a glycoprotein's half-life has been modified by pharmacokinetic analysis, as described in example 3 of the specification. Isolation and characterization of a glycoprotein having a desired O-linked carbohydrate modification from a cultured transgenic cell would not be substantially different from the same analysis of a modification reaction performed *in vitro*. Thus, no undue experimentation is required to perform the claimed invention.

Based on the foregoing, applicants respectfully request withdrawal of this ground of rejection.

4. Claim Rejections - 35 USC 102(b)

The Examiner has rejected claims 1-11 and 13 pursuant to 35 USC 102(b) as allegedly being anticipated by Wolff *et al.* (Protein Expression and Purification 22:414-421 (2001)). The Examiner asserts that Wolff teaches differences between native and recombinant molecules in terms of their glycosylation and the importance of reduced O-glycosylation in hereditary diseases. *See* paragraph bridging pp. 5-6 of the OA. The Examiner further asserts that Wolff indicates that engineered glycosylation pathways can be used to obtain recombinant inhibitors for clinical evaluation. *See* p. 6 of the OA. Based on the foregoing, the Examiner concludes that Wolff anticipates the claimed invention because the recombinant human C1 inhibitor reported in Wolff and the claimed C1 inhibitor are physically the same and must therefore have the same properties. *Id.* Applicants respectfully disagree.

Wolff reports an expression vector system utilizing insect cells for mass producing biologically active human recombinant C1 inhibitor for the treatment of hereditary deficiencies. *See* abstract. Wolff reports that thirteen glycosylation sites have been identified on the human C1 inhibitor protein (*see* p. 415, col. 1, first full paragraph), but does not discuss or otherwise opine on the significance of modification to O-linked carbohydrates, particularly in the

context of changes to plasma circulatory half-life. Applicants cannot identify any such discussion in the paragraph cited by the Examiner (*i.e.*, p. 419, col. 2, 2nd paragraph), nor in any other portion of the reference. Moreover, Wolff's discussion of engineered glycosylation pathways (*see* p. 420, col. 1) is directed to the inability of insect cells, used in Wolff's expression system, to produce eukaryotic glycoproteins with complex N-linked glycans. *See* p. 419, col. 2, 2nd full paragraph. The discussion is not related to the modification of carbohydrates generally, nor to O-linked carbohydrates in particular. Furthermore, Wolff reports that both the native and recombinant forms of C1 inhibitor "contained similar amounts of O-glycans." *See* p. 419, col. 1, "Analysis of C1INH O-Glycans".

Although Wolff reports removal of O-glycans from the C1 inhibitor for analysis of the presence of O-glycan chains, release of the O-glycans is reportedly performed by contacting the C1 inhibitor protein with lithium hydroxide. *See* paragraph bridging pp. 416-417. Wolff does not report O-linked carbohydrate modification by *in vitro* incubation with an enzyme preparation comprising one or more O-linked carbohydrate modifying enzymes, nor does it report *in vivo* modification by co-expression of a recombinant C1 inhibitor and a recombinant O-linked carbohydrate modifying enzyme, as required by independent claim 1. Thus, for at least these reasons, Wolff does not anticipate the presently claimed invention.

Based on the foregoing, applicants respectfully request withdrawal of this ground of rejection.

5. Claim Rejections - 35 USC 103(a)

The Examiner has rejected claims 16-22 pursuant to 35 USC 103(a) as allegedly being unpatentable over Paulson et al (WO 98/31826), Schoenberger et al. (FEBS 314: 430-434 (1992)), Wolff et al. (Protein Expression and Purification 22: 414-421 (2001), and Glaser et al. (WO 92/03149) for the reasons of record set forth in the Office Action of June 1, 2007. *See* p. 6 of the current OA. The June 1, 2007 Office Action refers to the reasons of record set forth in the Office Action of April 19, 2006. *See* p. 7 of the June 1, 2007 OA.

In the Office Action of April 19, 2006 the Examiner asserted that Paulson teaches increasing plasma circulatory half-life of therapeutic proteins by modification of both N- and O-linked oligosaccharides of recombinant glycoproteins. However, the Examiner acknowledged that Paulson does not teach the importance of O-glycosylation. *See* paragraph bridging p. 7-8 of

the April 19, 2006 OA. The Examiner further asserted that Schoenberger teaches C1 inhibitor and the characterization of the carbohydrate moieties, as well as removal of sialic acid from native molecules. *See* p. 8, 2nd paragraph of the April 19, 2006 OA. The Examiner also asserted that Wolff teaches production and purification of recombinant C1 inhibitor and identifies the glycosylation sites in the human protein. *See* p. 8, 3rd paragraph of the April 19, 2006 OA. Finally, the Examiner asserted that Glaser teaches a method of treating thrombotic disease using a therapeutic protein that has been modified in the sugar residues of the O-linked glycosylation domain, including deletion of sugar moieties. *See* p. 8, 4th paragraph of the April 19, 2006 OA. In the current Office Action, the Examiner indicates that the rejection has been restricted to "broad claims encompassing all protein in general," and that the generic methods described in Paulson for glycoproteins make the reference applicable to the "modulation of O-glycosylation of proteins" in general. *See* p. 7 of the current OA.

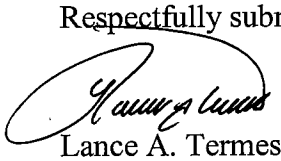
Independent claim 16 is directed to a method for extending blood circulatory half-life of a glycoprotein or of a glycoprotein comprising compound via the removal of one or more non-sialylated O-linked carbohydrates from the glycoprotein. None of the references reports or suggests that removal of a non-sialylated O-linked carbohydrate from a glycoprotein extends the blood circulatory half-life of the glycoprotein or a glycoprotein comprising compound, as claimed. Thus, claim 16, and dependent claims 17-22, would not have been obvious as of the priority date of the application.

As the Examiner acknowledges, Paulson does not recognize the importance of O-linked carbohydrate modification to extending the blood circulatory half-life of a glycoprotein. Rather, Paulson reports that blood circulatory half-life of glycoproteins is "highly dependent on the composition and structure of its *N-linked* carbohydrate groups." *See* p. 1, lines 16-17. Because nothing in the prior art suggests that modification of O-linked carbohydrates has an impact on the circulatory half-life of a glycoprotein, no motivation exists for combining Paulson with any reference reporting removal of non-sialylated O-linked carbohydrates to arrive at the claimed invention. Neither Wolff nor Schoenberger discusses extending circulatory half-life, and Glaser reports increasing circulatory half-life of a thrombomodulin analog by removing all or most of the sugar moieties in the 6 EGF-like domains (*see* p. 4, lines 35-38), which Glaser identifies as distinct from the O-linked glycosylation domain. *See* p. 11, lines 33-34. Thus, no

combination of the references reports or suggests that removal of non-sialylated O-linked carbohydrates can extend blood-circulatory half-life, as claimed. Because the prior art does not recognize that removal of non-sialylated O-linked carbohydrates can extend the blood circulatory half-life of a glycoprotein, as claimed, and because the Examiner has not set forth any argument identifying how this element would have been obvious to the skilled artisan as of the priority date of the application, a *prima facie* case of obviousness has not been established, and this ground of rejection should be withdrawn.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-326-2400.

Respectfully submitted,



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MINI REVIEW

1994, the year of sialyltransferases

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Introduction

Sialic acids are a family of closely related nine-carbon carboxylated sugars found at the non-reducing terminal position of mammalian cell surface sugar chains of glycoproteins, glycolipids and oligosaccharides. They are glycosidically linked to either the 3- or 6-hydroxyl groups of galactose residues (Gal), or to the 6-hydroxyl group of *N*-acetylglucosamine (GlcNAc) or *N*-acetylgalactosamine (GalNAc) residues, and can form polysialic chains via their 8-hydroxyl group and terminate with a sialic acid branched via the 8- or 9-hydroxyl group.

Because of their terminal position and their charge, sialylated oligosaccharide sequences have long been predicted to be information-containing molecules and critical determinants in cell–cell recognition processes (Rademacher *et al.*, 1988) and, indeed, for a very long time, cell surface sialic acid residues have been known to be involved as receptors for influenza virus (Rogers *et al.*, 1986). There are few recent examples in which these functions have been clearly defined, the best documented example being that of the selectin family. The selectins found on the endothelium (E- and P-selectins), platelets (P-selectin) and leukocytes (L-selectin) can bind to ligands that include oligosaccharide structures such as sialyl-Lewis^x (SLe^x), NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAcβ1-R (Foxall *et al.*, 1992).

Sialyltransferases, listed in Table I, are a subset of the glycosyltransferase family that use CMP-NeuAc as the activated sugar donor to catalyse the transfer of sialic acid residues to terminal non-reducing positions of oligosaccharide chains of glycoproteins and glycolipids (Sadler, 1984; Kornfeld and Kornfeld, 1985). Since sialyltransferases share the same sugar donors and recognize identical acceptor substrate, it was expected that they would exhibit similar protein sequences. Surprisingly, amino acid sequences of the cloned sialyltransferase cDNAs showed only very little homology with the exception of the short consensus sequence called the

Table I. The sialyltransferase family. (A) J.C.Paulson nomenclature; (B) J.T.Y.Lau nomenclature; (C) S.Tsuji nomenclature; (D) abbreviations currently used for the sialyltransferases acting on gangliosides. The sialic acid residue is transferred in the bold character sugar

Sialyltransferase full name	EC no.	Abbreviations				
		A	B	C	D	Others
α2,6-Sialyltransferase = α2,6-ST						
1 CMP-sialic acid: **Gal**β1-4GlcNAc α2,6-sialyltransferase	2.4.99.1	ST6(N)	SiaT-1	ST6Gal		ST-1
2 CMP-sialic acid: sialylα2-3Galβ1-3**GlcNAc**β α2,6-sialyltransferase	2.4.99.-			ST6GlcNAc		
3 CMP-sialic acid: R[a]-**GalNAc**α1-Ser(Thr) α2,6-sialyltransferase	2.4.99.3	ST6(O)-I		ST6GalNAc-I		
4 CMP-sialic acid: R[b]-Galβ1-3**GalNAc**α1-Ser(Thr) α2,6-sialyltransferase	2.4.99.-			ST6GalNAc-II		
5 CMP-sialic acid: sialylα2-3Galβ1-3**GalNAc**α1-Ser(Thr) α2,6-sialyltransferase	2.4.99.7	ST6(O)-II		ST6GalNAc-III		STY
α2,3-Sialyltransferase = α2,3-ST						
6 CMP-sialic acid: **Gal**β1-3/4GlcNAc-R α2,3-sialyltransferase	2.4.99.6	ST3(N)		ST3Gal-II		ST3GalB, ST-3
7 CMP-sialic acid: **Gal**β1-3/4GlcNAcβ-R α2,3-sialyltransferase	2.4.99.-					ST-4
8 CMP-sialic acid: **Gal**β1-4GlcNAc-R α2,3-sialyltransferase	2.4.99.-	ST3(N)-II				
9 CMP-sialic acid: **Gal**β1-3GalNAcβ-R α2,3-sialyltransferase	2.4.99.4	ST3(O)-I	SiaT-4a	ST3Gal-Ia		SAT-4, ST-2, ST3GalA.1
10 CMP-sialic acid: **Gal**β1-3GalNAc α2,3-sialyltransferase	2.4.99.-	ST3(O)-II	SiaT-4b	ST3Gal-Ib		ST3GalA.2
11 CMP-sialic acid: **Gal**β1-4GlcNAc or **Gal**β1-3GalNAc α2,3-sialyltransferase	2.4.99.-	ST3(O/N)	SiaT-4c	ST3Gal-III		SAT-3, ST3GalC, STZ
12 CMP-sialic acid: **Gal**β1-4Glcβ1-O-Cer α2,3-sialyltransferase	2.4.99.9				SAT-I	G_{M3} synthase
13 CMP-sialic acid: **Gal**β1-3GalNAcβ1-4[R[c]-]Galβ1-4Glcβ1-O-Cer α2,3-sialyltransferase	2.4.99.2				SAT-IV	G_{D1b} synthase
α2,8-Sialyltransferase = α2,8-ST						
14 CMP-sialic acid: **NeuAc**α2-3Galβ1-4GlcNAc-R α2,8-sialyltransferase	2.4.99.-	STX		ST8Sia-II		α2,8-ST
15 CMP-sialic acid: NeuAcα2-8**NeuAc**α2-3Galβ1-4GlcNAc-R α2,8-sialyltransferase	2.4.99.-			ST8Sia-III		
16 CMP-sialic acid: NeuAcα2-8NeuAcα2-3Galβ1-4GlcNAc-R α2,8-sialyltransferase	2.4.99.-			ST8Sia-IV		polysialyl α2,8-ST, PST-1
17 CMP-sialic acid: sialylα2-3Galβ1-4Glcβ1-O-Cer α2,8-sialyltransferase	2.4.99.8			ST8Sia-I	SAT-II	G_{D3} synthase
18 CMP-sialic acid: sialylα2-8sialylα2-3Galβ1-4Glcβ1-O-Cer α2,8-sialyltransferase	2.4.99.-				SAT-III	G_{T3} synthase
19 CMP-sialic acid: NeuAcα2-3Galβ1-3GalNAcβ1-4[R[c]-]Galβ1-4Glcβ1-O-Cer α2,8-sialyltransferase	2.4.99.-				SAT-V	

[a]R = H-, Galβ1-3, sialylα2-3Galβ1-3.
[b]R = H-, sialylα2-3.
[c]R = H-, sialylα2-3, sialylα2-8sialylα2-3, sialylα2-8sialylα2-8sialylα2-3.

Table II. Various forms of sialylated terminal sequences occurring in mammalian glycoproteins and glycolipids

Structure	Origin	References
NeuAcα2-3Galβ1-4GlcNAcβ	Ubiquitous	Montreuil, 1980
NeuAcα2-3Galβ1-3GlcNAcβ	Ubiquitous	Montreuil, 1980
NeuAcα2-6Galβ1-4GlcNAcβ	Ubiquitous	Montreuil, 1980
NeuAcα2-3Galβ1-3GlcNAcβ NeuAcα2-6	Bovine and rat glycoproteins	Mizuochi *et al.*, 1979 Yoshima *et al.*, 1981
NeuAcα2-6GalNAcβ1-4GlcNAcβ	Pituitary hormones Bowes melanoma tPA Bovine lactotransferrin CD 36	Weisshaar *et al.*, 1991 Chan *et al.*, 1991 Coddeville *et al.*, 1992 Nakata *et al.*, 1991
(NeuAcα2-8)$_n$[a] NeuAcα2-3Galβ1-3/4GlcNAcβ	N-CAM, postnatal rat kidney, heart and muscle α-subunit of sodium channel	Troy, 1992 Zuber *et al.*, 1992
NeuAcα2-9NeuAcα2-3/6Galβ1-4GlcNAcβ	Human PA1 embryonal carcinoma cells	Fukuda *et al.*, 1985
NeuAcα2-6GalNAcα1-Ser/Thr	Ubiquitous	Schachter and Brockhausen, 1989
NeuAcα2-3Galβ1-3GalNAcα1-Ser/Thr[b]	Ubiquitous	Schachter and Brockhausen, 1989
NeuAcα2-3Galβ1-3GalNAcα1-Ser/Thr NeuAcα2-6	Ubiquitous	Schachter and Brockhausen, 1989
NeuAcα2-3Gal-Cer (GM_4)	Human and avian brain	Ledeen, 1978; Kundu, 1992
NeuAcα2-3[R_1][c] Galβ1-4Glc-Cer	Extra neuronal tissue, erythrocytes, plasma	Ledeen, 1978; Kundu, 1992
NeuAcα2–8NeuAcα2-3[R_1]Galβ1-4Glc-Cer	Extra neuronal tissue, erythrocytes, plasma, human brain	Ledeen, 1978; Wiegandt, 1982; Kundu, 1992
NeuAcα2-8NeuAcα2-8NeuAcα2-3[R_1]Galβ1-4Glc-Cer	Pig adipose tissue	Ledeen, 1978; Kundu, 1992
NeuAcα2-3Galβ1-3GalNAcβ1-R_2[d]	Human brain	Ledeen, 1978; Wiegandt, 1982; Kundu, 1992
NeuAcα2-8NeuAcα2-3Galβ1-3GalNAcβ1-R_2	Bovine, human and chicken brain	Ledeen, 1978; Wiegandt, 1982; Kundu, 1992

[a] n from 1 to up to 55.
[b] May be sulphated in 6 position to GalNAc.
[c] R_1 = H (G_{M3}), or GalNAcβ1-4 (G_{M2}), or Galβ1-3GalNAcβ1-4 (G_{M1a}).
[d] R_2 = LacCer, or G_{M3}, or G_{D3}, or G_{T3}.

sialylmotif (Drickamer, 1993; Livingston and Paulson, 1993). However, sialyltransferases share a common domain structure with the other glycosyltransferases. They are Type II transmembrane glycoproteins with a short NH_2-terminal cytoplasmic tail, a 16–20 amino acid signal anchor domain and an extended stem region followed by a large COOH-terminal catalytic domain (Paulson and Colley, 1989).

The subcellular localization of the sialyltransferases has been extensively studied. These enzymes have been shown to be restricted to the trans cisternae of the Golgi apparatus and the trans Golgi network. However, some studies have suggested that these enzymes may have a more diffuse distribution throughout the Golgi apparatus in some cells (Berger and Hesford, 1985; Roth, 1987). In spite of the fundamental role played by sialyltransferases in the biosynthesis of specific sialylated sequences to date, there is limited information available on their protein structure, mechanism of enzyme action and on the cellular mechanisms involved in the regulation of their transcriptional expression.

The purpose of this two-part review is to cover the recent progress in sialyltransferase enzymology afforded by molecular biology and cloning techniques. In the first part, classical purification procedures from mammalian and avian sources that have been widely used to characterize the enzyme activity and their acceptor substrates will be presented. Because of the low cellular levels of these membrane-bound enzymes, these procedures have rarely resulted in peptide sequence information sufficient to clone and sequence the respective cDNAs or to raise specific antibodies. The first section also compares and classifies the sialyltransferases acting on glycoproteins and glycolipids. The second part describes the approaches that have proven useful to isolate the cDNA clones and which include protein enzyme purification, gene transfer, cross-hybridization methods and the polymerase chain reaction (PCR). Finally, as more cDNAs become available, it is possible to gather the recent inferences on transcriptional regulation of sialyltransferase gene expression.

Sialylated structures occurring in mammalian glycoconjugates

During these past few years, there has been a large increase in the number of sialylated structures described and a myriad of novel glycan structures have been characterized in living organisms. Table II lists the main sialylated oligosaccharide sequences found in the glycoproteins or glycolipids of mammals.

The tremendous structural diversity of sialylated terminal sequences derives, first, from variations in the linkages and in the number of sialic acid residues. As an example, the structure Galβ1-4GlcNAc can be sialylated either on the 3- or the 6-hydroxyl group of the galactose residue. In addition, the carbohydrate component of neural cell adhesion molecules (N-CAMs) is made up exclusively of α2-8-linked

N-acetylneuraminic acid with a degree of polymerization as high as 55 residues (Troy, 1992). Second, structural variations also arise from various chemical structures of the sialic acid due to the presence of *N*-acetyl or *N*-glycoloyl neuraminic acid and up to three substitutions (acetyl, lactoyl and methyl) on their 4-, 7-, 8- or 9-hydroxyl groups (not shown in Table II). Finally, the internal oligosaccharide structure can be further modified by sulphation or fucosylation, modifications which are also not taken into account in Table II.

The sialyltransferase family

The α2,6-sialyltransferase sub-family

Sialic acid α2,6-linked to Gal

*CMP-sialic acid: **Galβ1-4GlcNAc** α2,6-sialyltransferase (EC 2.4.99.1; ST6(N); # 1) (where the acceptor sugar is in bold characters and numbers refer to sialyltransferases quoted in Table I).* The ST6(N) enzyme catalyses the following reaction:

CMP-sialic acid + **Galβ1-4GlcNAc**-R → sialylα2-6**Galβ1-4GlcNAc**-R + CMP

The ST6(N) enzyme is the only sialyltransferase able to transfer sialic acid to the 6-hydroxyl group of the galactose residue at the terminal position of the disaccharide Galβ1-4GlcNAc, where the acceptor may be a free disaccharide or may represent a non-reducing terminal *N*-acetyllactosamine unit on an N- or O-linked oligosaccharide. The enzyme is highly specific for Type 2 oligosaccharide chains (Galβ1-4GlcNAc) and is unable to transfer sialic acid onto Type 1 oligosaccharide chain (Galβ1-3GlcNAc) or onto the T-antigen (Galβ1-3GalNAc) (Weinstein *et al.*, 1982b). However, in *in vitro* assays, the ST6(N) enzyme transfers sialic acid onto other oligosaccharide structures, such as lactose (Galβ1-4Glc; Paulson *et al.*, 1977b), GalNAcβ1-4GlcNAc (Nemansky and van den Eijnden, 1992) or Manβ1-4GlcNAc (Van Pelt *et al.*, 1989). In fact, a precise determination of the acceptor specificity using synthetic modified Type 2 acceptors indicates that ST6(N) requires the 6-hydroxyl group of the β-Gal and the 2-acetamido group of the GlcNAc, and that most of the other hydroxyl groups accept modifications (Wlasichuk *et al.*, 1993).

Type 2 terminal sequences occur mainly in complex *N*-glycan chains. Joziasse *et al.* (1985b, 1987) have shown that ST6(N) preferentially sialylates the Gal residue at the Galβ1-4GlcNAcβ1-2Manα1-3 branch rather than the Galβ1-4 GlcNAcβ1-2Manα1-6 branch in a biantennary structure, and the presence of an additional Galβ1-4GlcNAcβ1-6Manα1-6 branch in tri- and tetra-antennary glycans strongly weakens the rate of transfer onto both branches. The ST6(N) enzyme is widely expressed in a number of tissues and cells, especially in liver where it participates in the sialylation of serum glycoproteins. The enzyme has been purified to homogeneity from different animal livers and hepatoma cells (Bendiak and Cook, 1982; Miyagi and Tsuiki, 1982; Weinstein *et al.*, 1982a; Sticher *et al.*, 1991; see Table III). A soluble form of the enzyme has also been purified from bovine colostrum (Paulson *et al.*, 1977a; Hesford *et al.*, 1984) and was shown to arise from the membrane-bound enzyme by the proteolytic cleavage of the non-catalytic N-terminal part of the polypeptide that contains the membrane signal anchor. Antibodies raised against the rat liver enzyme were used to screen a λgt11 cDNA library leading to the cloning of the rat liver enzyme (Weinstein *et al.*, 1987). A soluble form of ST6(N) is also present in serum (Kaplan *et al.*, 1983; Van Dijk *et al.*, 1985; Lammers and Jamieson, 1989a) and its level is modulated according to physiological or pathological conditions (cirrhosis, pregnancy, cancer, etc.). It is increased during the acute-phase response of the liver to tissue injury. The release of ST6(N) from liver to serum involves a cathepsin D-like protease which is induced during the inflammatory response (Lammers and Jamieson, 1989b) and ST6(N) can be considered in these terms as an acute-phase protein like α_1-acid glycoprotein (Jamieson *et al.*, 1993).

Sialic acid α2,6-linked to GalNAcα1-Ser(Thr)

*CMP-sialic acid: R-**GalNAcα1-Ser(Thr)** α2,6-sialyltransferase (EC 2.4.99.3; ST6(O)-I; # 3).* The ST6(O)-I enzyme catalyses the following reaction:

CMP-sialic acid + R-**GalNAcα1-Ser(Thr)** → R-[sialylα2-6]-**GalNAcα1-Ser(Thr)** + CMP

where R can be H-, Galβ1-3- or sialylα2-3Galβ1-3-. This sialyltransferase has been highly purified from porcine submaxillary glands (Rearick *et al.*, 1979; Sadler *et al.*, 1979a,b) and is present in many mucin-secreting tissues. It appears to be absent from human, porcine, rat and canine liver tissues (van den Eijnden *et al.*, 1981). This enzyme also must recognize the peptide aglycon structure as well as the carbohydrate structure for acceptor recognition (Bergh and van den Eijnden, 1983) and has been shown to act poorly on Fucα1-2Galβ1-3GalNAcα1-Ser. Therefore, the preferential pathway for the synthesis of Fucα1-2Galβ1-3[sialylα2-6]GalNAcα1-Ser, a component of several mucins, involves first ST6(O)-I followed by the action of α2-fucosyltransferase (Beyer *et al.*, 1981). ST6(O)-I is responsible for the biosynthesis of the sialylTn antigen (sialyl α2-6GalNAcα1-Ser/Thr) and of the sialylα6-T antigen (Galβ1-3[NeuAcα2-6]GalNAcα1-Ser/Thr), both occurring at the cell surface of tumour cells (Hakomori *et al.*, 1984).

*CMP-sialic acid: sialylα2-3Galβ1-3**GalNAcα1-Ser(Thr)** α2,6-sialyltransferase (EC 2.4.99.7; ST6(O)-II; # 5).* The ST6(O)-II enzyme catalyses the following reaction:

CMP-sialic acid + sialylα2-3Galβ1-3**GalNAcα1-Ser(Thr)** → sialylα2-3Galβ1-3[sialylα2-6]**GalNAcα1-Ser(Thr)** + CMP

This enzyme was characterized in fetal calf liver (Bergh *et al.*, 1983), in ovine and porcine submaxillary glands (Bergh and van den Eijnden, 1983), and in rat brain (Baubichon-Cortay *et al.*, 1986). In contrast to the porcine submaxillary gland ST6(O)-I described above, which can act on GalNAc-protein, Galβ1-3GalNAc-protein and sialylα2-3Galβ1-3GalNAc-protein, the ST6(O)-II enzyme from fetal calf liver microsomes acts only on the sialylα2-3Galβ1-3GalNAc-protein acceptor. In addition, purified salivary gland ST6(O)-I does not transfer sialic acid onto the *p*-nitrophenyl derivatives of GalNAc, Galβ1-3GalNAc and sialylα2-3Galβ1-3GalNAc, whereas fetal calf liver ST6(O)-II can transfer sialic acid in α2-6 linkage to the GalNAc residue of sialylα2-3Galβ1-3GalNAc-R where R can be either a protein or a hydrophobic group. Furthermore, the ST6(O)-II enzyme from rat brain was shown to be selectively inhibited by *N*-ethylmaleimide (Baubichon-Cortay *et al.*, 1986). This enzyme, also termed ST6GalNAc-III, has recently been cloned from rat brain by Sjoberg *et al.* (1995).

Table III. Sialyltransferases characterized and/or purified in mammalian and avian tissues or cells

Number[a]	cDNA cloned	Linkage synthesized[b]	EC number	Origin	MW (kDa)	References
1	yes	**NeuAcα2-6**Galβ1-4GlcNacβ	2.4.99.1	bovine colostrum (purified)	56/43	Paulson *et al.* (1977)
				bovine colostrum (purified)	76/54	Hesford *et al.* (1984)
				rat liver (purified)	47	Weinstein *et al.* (1982)
				embryonic chicken liver (purified)		Bendiak and Cook (1982)
				rat liver and hepatomas (purified)	37/43	Miyagi and Tsuiki (1982)
				human liver (purified)		Sticher *et al.* (1991)
2	not cloned	**NeuAcα2-6** \ GlcNAcβ-R / NeuAcα2-3Galβ1-3	2.4.99.-	rat liver		Paulson *et al.* (1984)
				rabbit, rat, fetal calf liver, human liver and placenta		de Heij *et al.* (1986)
3	yes	**NeuAcα2-6**GalNAcα-Ser/Thr	2.4.99.3	porcine submaxillary glands (purified)	160	Sadler *et al* (1979b)
5	yes	**NeuAcα2-6** \ GalNAcα-Ser/Thr / NeuAcα2-3Galβ1-3	2.4.99.7	fetal calf liver		Bergh *et al.* (1983)
						Bergh and van den Eijnden (1983)
				ovine and porcine submaxillary glands		Bergh and van den Eijnden (1983)
6	yes	**NeuAcα2-3**Galβ1-3(4)GlcNAcβ	2.4.99.6	rat liver	56	Weinstein *et al* (1982)
				human colorectal carcinoma cells (purified)		Liepkans *et al.* (1988)
8	not cloned	**NeuAcα2-3**Galβ1-4GlcNAcβ	2.4.99.-	human placenta		Van den Eijnden and Schiphorst (1981)
						Nemansky *et al.* (1992)
						Nemansky and van den Eijnden (1993)
				human liver		Nemansky *et al.* (1992)
9	yes	**NeuAcα2-3**Galβ1-3GalNAcβ-R	2.4.99.4	porcine submaxillary glands (purified)	49/44	Sadler *et al.* (1979b)
				human placenta (purified)		Rearick *et al.* (1979)
				porcine liver (purified)	65	Joziasse *et al* (1985)
					40/50	Conradt *et al.* (1988)
11	yes	**NeuAcα2-3**Galβ1-4GlcNAc **NeuAcα2-3**Galβ1-3GlcNAc	2.4.99.-	embryonic chicken brain human lung carcinoma cells		Basu *et al* (1982) Holmes *et al.* (1986)
12	not cloned	**NeuAcα2-3**Galβ1-4Glcβ1-O-Cer	2.4.99.9	rat liver (purified)	60	Melkerson-Watson and Sweeley (1991)
				rat brain (purified)		Preuss *et al.* (1993)
13	not cloned	**NeuAcα2-3**Galβ1-3GalNacβ1-4R_1[c]	2.4.99.2	embryonic chicken brain (purified)		Basu *et al.* (1988)
				rat brain (purified)	44	Gu *et al.* (1990a)
14	yes	**NeuAcα2-8**NeuAcα2-3Galβ1-4GlcNAc	2.4.99.-	human neuroblastoma cells (purified)		Stoykova and Glick (1995)
16	yes	**NeuAcα2-8**NeuAcα2-8NeuAcα2-*R*	2.4.99.-	fetal rat brain		McCoy *et al* (1985)
				embryonic chick brain (purified)		Sevigny *et al.* (1995)
17	yes	**NeuAcα2-8**NeuAcα2-3Galβ1-4Glcβ1-O-Cer	2.4.99.8	rat brain (purified)		Gu *et al* (1990b)
19	not cloned	**NeuAcα2-8**NeuAcα2-3Galβ1-3GalNAcβ1-4R_1	2.4.99.-	rat liver		Iber *et al.* (1992); Van Echten and Sandhoff (1993)

[a]The numbers of sialyltransferases correspond to those in Table I.
[b]The sialic acid residue transferred is in bold letters.
[c]R_1 = LacCer, or G_{M3}, or G_{D3}, or G_{T3}.

It is clear that there are at least two distinct α2,6-sialyltransferases involved in the synthesis of α2-6GalNAc linkage in *O*-glycans. One enzyme (ST6(O)-I; EC 2.4.99.3) is probably involved in mucin synthesis in many mucin-secreting tissues, but is absent in fetal and adult mammalian liver. The other (ST6(O)-II; EC 2.4.99.7) is more widely distributed, being present in fetal calf liver, porcine and ovine submaxillary glands (Bergh and van den Eijnden, 1983; Bergh *et al.*, 1983). This enzyme is probably involved in the synthesis of the tetrasaccharide sialylα2-3Galβ1-3[sialylα2-6]GalNAc in non-mucin glycoproteins.

CMP-sialic acid: R-Galβ1-3GalNAcα1-Ser(Thr) α2,6-sialyltransferase (EC 2.4.99.-; ST6GalNAc-II; # 4). A third enzyme, able to transfer sialic acid to the 6-hydroxyl group of GalNAcα1-Ser(Thr), was recently cloned from a chicken testis cDNA library and expressed in COS-7 cells (Kurosawa *et al.*, 1994).

The avian enzyme catalyses the following reaction:

CMP-sialic acid + R-Galβ1-3**GalNAc**α1-Ser(Thr) → R-Galβ1-3[sialylα2-6]**GalNAc**α1-Ser(Thr) + CMP

where R can be H- or sialylα2-3. Its specificity differs slightly from both mammalian ST6(O)-I and ST6(O)-II by the fact that, in contrast to ST6(O)-I, the avian enzyme cannot act on Tn antigen (GalNAcα1-Ser/Thr), but can act on the T antigen (Galβ1-3GalNAcα1-Ser/Thr) which is not an acceptor substrate for ST6(O)-II. In addition, the chicken enzyme strictly requires a polypeptide aglycon for its activity (Kurosawa *et al.*, 1994) since Galβ1-3GalNAcα1-benzyl, asialo-G_{M1} (Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-O-Cer; where Cer is ceramide) and G_{M1b} (NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-O-Cer) are not acceptor structures in *in vitro* assays.

Sialic acid α2,6-linked to GalNAcβ1-4GlcNAc

The NeuAcα2-6**GalNAc**β1-4GlcNAc oligosaccharide sequence (Table II) has been identified in the terminal position of N-linked glycans from several mammalian glycoproteins, including pituitary hormones (Weisshaar *et al.*, 1991), CD 36 (Nakata *et al.*, 1991) and bovine lactoferrin (Coddeville *et al.*, 1992). The enzyme catalysing this linkage is not yet characterized, but some evidence indicates that ST6(N) (# 1)

in bovine colostrum is responsible for the synthesis of this unusual terminal sialylated sequence occurring in bovine milk glycoproteins (Nemansky and van den Eijnden, 1992).

Sialic acid α2,6-linked to GlcNAc

CMP-sialic acid: sialylα2-3Galβ1-3GlcNAcβ-R α2,6-sialyltransferase (EC 2.4.99.-; # 2). This enzyme catalyses the following reaction:

CMP-sialic acid + sialylα2-3Galβ1-3**GlcNAc**β-R →
sialylα2-3Galβ1-3[sialylα2-6]**GlcNAc**β-R + CMP

where R is a complex-type N-linked glycan. This sialyltransferase has been characterized in rat liver (Paulson *et al.*, 1984; de Heij *et al.*, 1986), in rabbit, human and fetal calf liver, and human placenta (de Heij *et al.*, 1986). It is involved in the biosynthesis of the terminal sialylα2-3Galβ1-3[sialylα2-6]-GlcNAcβ1-R group occurring in human milk oligosaccharides and in the glycan chains of several *N*-glycoproteins. This enzyme prefers sialylα2-3Galβ1-3GlcNAcβ1-R as an acceptor substrate and shows very low or no activity towards Galβ1-3GlcNAcβ1-R or GlcNAcβ1-R acceptor structures, respectively. Finally, the biosynthesis of the disialylated sequences first requires the action of the Galβ1-3/4GlcNAc α-2,3-sialyltransferase (# 6) prior to α-2,6 sialylation of GlcNAc residues.

The α2,3-sialyltransferase sub-family

Sialic acid α2,3-linked to Galβ1-3GalNAc

*CMP-sialic acid: **Gal**β1-3GalNAcβ-R α2,3-sialyltransferase (EC 2.4.99.4; ST3(O)-I; # 9).* This enzyme catalyses the following reaction:

CMP-sialic acid + **Gal**β1-3GalNAc-R →
sialylα2-3**Gal**β1-3GalNAc-R + CMP

This enzyme has been purified to homogeneity from porcine submaxillary glands (Rearick *et al.*, 1979; Sadler *et al.*, 1979a,b), from human placenta (Joziasse *et al.*, 1985a) and from porcine liver (Conradt *et al.*, 1988). The same enzymatic activity has also been reported in ovine submaxillary glands (Bergh *et al.*, 1982), rat brain (Baubichon-Cortay *et al.*, 1989), human colon carcinoma cells HT-29 (Dall'Olio *et al.*, 1993; Huet *et al.*, 1995), Ehrlich tumour cells (Shigeta *et al.*, 1994) and must be present in many other tissues to achieve the synthesis of sialylα3-T antigen. This enzyme has also been characterized in FR3T3 rat fibroblasts, and its expression is decreased 4-fold after cells are transfected with the c-Ha-*ras* oncogene (Delannoy *et al.*, 1993).

The purified enzyme catalyses the incorporation of sialic acid into glycoproteins, glycolipids and oligosaccharides which possess a terminal Galβ1-3GalNAc unit (i.e. various asialomucins, antifreeze glycoprotein, asialofetuin, ganglioside G_{M1}, etc.). The enzyme is also able to transfer sialic acid onto the 3-hydroxyl group of a Gal residue of Galβ1-3GalNAc α-linked to phenyl (Klohs *et al.*, 1981), benzyl, *p*- and *o*-nitrophenyl, but Galβ1-3GalNAc β-linked to benzyl is a poor acceptor substrate (Kuhns *et al.*, 1993). Lactose (Galβ1-4Glc), as well as oligosaccharides containing terminal Galβ1-3GlcNAc, or Galβ1-4GlcNAc, are also poor acceptors. ST3(O)-I does not act on lactosylceramide (Galβ1-4Glcβ1-O-Cer). The addition of sialic acid to the lactosylceramide (sialylα2-3Galβ1-4Glcβ1-O-Cer) requires another α2,3-sialyltransferase (EC 2.4.99.9; SAT-I; # 12). The activity of the porcine submaxillary gland ST3(O)-I has been shown to be modulated by the binding of detergents or lipids (Westcott *et al.*, 1985).

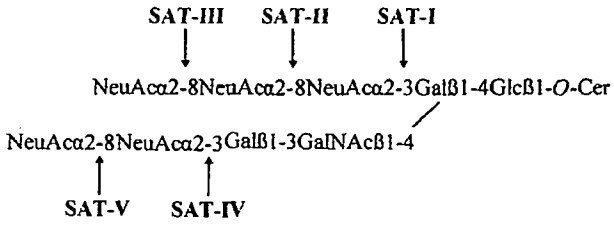


Fig. 1. Schematic representation of the sialyltransferases involved in the biosynthesis of gangliosides. Sialyltransferases are named SAT-I to SAT-V according to the current denomination used for gangliosides (Van Echten and Sandhoff, 1993).

ST3(O)-I from human placenta has a 20-fold lower affinity in terms of K_m for the donor substrate CMP-NeuAc than the corresponding porcine submaxillary gland enzyme (Joziasse *et al.*, 1985a) and both enzymes present differences in specific activities toward antifreeze glycoprotein. This indicates that the human placental enzyme and the porcine submaxillary gland ST3(O)-I enzyme are not identical. ST3(O)-I is also found in normal granulocytes, it is active in leukaemia-derived cell line and its activity is increased 3-fold in leukaemia cells (Baker *et al.*, 1987; Kanani *et al.*, 1990). The enzyme was also increased in breast cancer cells compared to normal mammary epithelial cells (Brockhausen *et al.*, 1995) and in colon cancer tissue compared to normal tissue (Yang *et al.*, 1994). Enzymes from human placenta and from myeloid leukaemia cells were compared and showed distinct acceptor substrate specificity differences, although both enzymes have an absolute requirement for the 3-hydroxyl group of the β-Gal residue of Galβ1-3GalNAcα-benzyl (Kuhns *et al.*, 1993).

The differences in substrate specificities of enzymes characterized or purified from different sources predicted that more than one enzyme was able to transfer sialic acid in α2,3 linkage to the Galβ1-3GalNAc sequence, and the recent cloning and expression of **Gal**β1-3GalNAc-R α-2,3-sialyltransferase from different cDNA libraries described below have confirmed the fact that at least two different enzymes are expressed in mammals.

CMP-sialic acid: Galβ1-3GalNAcβ1-4[R-]Galβ1-4Glcβ1-O-Cer α2,3-sialyltransferase (EC 2.4.99.2; SAT-IV; # 13) (see Figure 1 for the nomenclature of the sialyltransferases involved in the biosynthesis of gangliosides). As represented in Figure 1, SAT-IV catalyses the transfer of sialic acid to the 3-hydroxyl group of the terminal Gal residue on G_{A1}, G_{M1a}, G_{D1b} or G_{T1c} (Pohlentz *et al.*, 1988; Seyfried *et al.*, 1994; Freischütz *et al.*, 1995). The enzyme has been purified from embryonic chicken brain (Basu *et al.*, 1988) and from rat brain (Gu *et al.*, 1990a). However, it is not clear to date whether the enzyme differs from those acting on both glycoproteins and glycolipids previously described (ST3(O)-I, # 9; ST3(O/N), # 11; or ST3(O)-II, # 10). Indeed, the acceptor substrate specificity of the so-called SAT-IV has only been determined using glycolipidic acceptor substrates and, even if the enzyme has been purified to homogeneity, to date there is no published information available on its amino acid sequence. Therefore, SAT-IV cannot be compared in terms

of acceptor substrate specificity or sequence homologies with the other Galβ1-3GalNAc α2,3-sialyltransferases.

Sialic acid α2,3-linked to Galβ1-3/4GlcNAc

*CMP-sialic acid: **Galβ1-4GlcNAc** or **Galβ1-3GalNAc** α2,3-sialyltransferase (EC 2.4.99.-; ST3(O/N); # 11).* The ST3(O/N) enzyme catalyses the following two reactions:

CMP-sialic acid + **Gal**β1-4GlcNAc-R → sialylα2-3**Gal**β1-4GlcNAc-R + CMP

and

CMP-sialic acid + **Gal**β1-3GalNAc-R → sialylα2-3**Gal**β1-3GalNAc-R + CMP

The ST3(O/N) enzyme has not been purified to date. However, it has been characterized in embryonic chicken brain (Basu *et al.*, 1982) and in human lung carcinoma PC9 cells (Holmes *et al.*, 1986). The enzyme is able to transfer sialic acid onto the 3-hydroxyl group of the terminal Gal residue of paraglobo-side Lc*n*OSe$_4$-Cer (**Gal**β1-4GlcNAcβ1-3Galβ1-4Glcβ1-O-Cer) to form the L_{M1} ganglioside (sialylα2-3**Gal**β1-4GlcNAc-β1-3Galβ1-4Glcβ1-O-Cer). The enzyme from embryonic chicken brain also catalyses the transfer of sialic acid onto the 3-hydroxyl group of terminal Gal residue of G_{A1} (**Gal**β1-3GalNAcβ1-4Galβ1-4Glcβ1-O-Cer) to form G_{M1b} (sialyl-α2-3**Gal**β1-3GalNAcβ1-4Galβ1-4Glcβ1-O-Cer), but with a 10-fold lower affinity in terms of K_m for G_{A1} than for Lc*n*OSe$_4$-Cer (Basu *et al.*, 1982). ST3(O/N) is the only known sialyl-transferase able to sialylate *in vitro* glycolipids containing a Galβ1-4GlcNAcβ- terminal sequence and therefore it is involved in the biosynthesis of the sialyl-Le^x determinant occurring in the Type 2 lactoseries (Holmes *et al.*, 1986). ST3(O/N) has recently been cloned from a human placenta cDNA library (Kitagawa and Paulson, 1994a).

*CMP-sialic acid: **Galβ1-3(4)GlcNAc** α2,3-sialyltransferase (EC 2.4.99.6; ST3(N); # 6).* The ST3(N) enzyme catalyses the following reaction:

CMP-sialic acid + **Gal**β1-3(4)GlcNAc-R → sialylα2-3**Gal**β1-3(4)GlcNAc-R + CMP

The ST3(N) enzyme has been purified to homogeneity from rat liver (Weinstein *et al.*, 1982a) and from human colorectal carcinoma cells (Liepkans *et al.*, 1988). Although this enzyme prefers Type 1 chain (Galβ1-3GlcNAc) acceptors, it also transfers sialic acid onto Type 2 acceptors (Galβ1-4GlcNAc), but with lower efficiency (Weinstein *et al.*, 1982b). The precise acceptor specificity was determined using synthetic modified Type 1 and Type 2 disaccharide acceptors, and indicates that rat liver ST3(N) requires the 3-, 4- and 6-hydroxyls of the terminal β-Gal, and some contribution from the subterminal sugar. This may explain the cross-reactivity of this enzyme for the Type 1 and Type 2 acceptors (Wlasichuk *et al.*, 1993). ST3(N) acts weakly on Galβ1-3[NeuAcα2-6]GlcNAcβ sequence (Chandra-sekaran *et al.*, 1995). As discussed above, this indicates that the main pathway leading to the disialyl tetrasaccharide sequence NeuAcα2-3Galβ1-3[NeuAcα2-6]GlcNAcβ first involves ST3(N) before the α2-6 sialylation of GlcNAc. In addition, ST3(N) is involved in the biosynthesis of sialyl-Le^x and sialyl-Le^a sequences, the levels of which are increased in tumour cells and carcinomas. It has been shown that ST3(N) does not act on the Le^a structure (Galβ1-3[Fucα1-4]GlcNAcβ-R), indicating that the α2-3 sialylation of the Gal residue precedes α1-4 fucosylation of GlcNAc during the formation of the sialyl-Le^a sequence (Chandrasekaran *et al.*, 1995). The cDNA encoding ST3(N) has been cloned from rat liver (Wen *et al.*, 1992) and human placenta (Kitagawa and Paulson, 1993) cDNA libraries.

*CMP-sialic acid: **Galβ1-4GlcNAc** α2,3-sialyltransferase (EC 2.4.99.-; ST3(N)-II; # 8).* The ST3(N)-II catalyses the following reaction:

CMP-sialic acid + **Gal**β1-4GlcNAc-R → sialylα2-3**Gal**β1-4GlcNAc-R + CMP

The ST3(N)-II enzyme has been characterized in fetal calf liver, embryonic chicken brain, human placenta and several other tissues (van den Eijnden and Schiphorst, 1981), and more recently in human liver (Nemansky *et al.*, 1992). In contrast to ST3(N) described above, which acts preferentially on the type chains (Galβ1-3GlcNAc), the ST3(N)-II enzyme from human placenta acts preferentially on Type 2 chains (Galβ1-4GlcNAc). The enzyme is also able to transfer sialic acid onto the 3-hydroxyl group of a Gal residue of Galβ1-3GalNAc sequence (Nemansky and van den Eijnden, 1993) and it is possible that ST3(N)-II corresponds to the ST3(O/N) enzyme recently cloned from a human placenta cDNA library by Kitagawa and Paulson (1994a).

Sialic acid α2,3-linked to Galβ1-4Glcβ1-O-Cer

*CMP-sialic acid: **Galβ1-4Glcβ1-O-Cer** α2,3-sialyltransferase (EC 2.4.99.9; SAT-I; G_{M3} synthase; # 12).* The G_{M3} synthase enzyme (see Figure 1) catalyses the following reaction:

CMP-sialic acid + **Gal**β1-4Glcβ1-O-Cer → sialylα2-3**Gal**β1-4Glcβ1-O-Cer + CMP

SAT-I was characterized in embryonic chicken brain (Kaufman *et al.*, 1966), in rat liver and cultured rat hepatocytes (Keenan *et al.*, 1974; Iber *et al.*, 1990; Mesaric and Decker, 1990), in rat brain (Ng and Dain, 1977) and in B16 melanoma cells (Tsuchiya *et al.*, 1993). SAT-I has been purified from rat liver (Melkerson-Watson and Sweeley, 1991) and from rat brain (Preuss *et al.*, 1993). The rat brain enzyme showed high specificity for lactosylceramide (Preuss *et al.*, 1993). However, even if the rat liver enzyme is effectively able to transfer sialic acid onto the 3-hydroxyl group of the terminal Gal residue of lactosylceramide (**Gal**β1-4Glcβ1-O-Cer), glucosylceramide (Glcβ1-O-Cer), galactosylceramide (Galβ1-O-Cer) and asialo-G_{M1} (Galβ1-3GalNAcβ1-4**Gal**β1-4Glcβ1-O-Cer) are also acceptor substrates for rat liver SAT-I, but to a lesser extent (Melkerson-Watson and Sweeley, 1991). G_{M3}, G_{M2}, G_{M1} and other sialyl-gangliosides as well as different asialoglyco-proteins are not acceptor substrates for SAT-I. A recent study using 19 molecular species of lactosylceramide has shown that the apparent K_m of the enzyme for LacCer varied 6-fold among these molecular species of lactosylceramide. The reaction mechanism was shown to be a sequential, ordered bi–bi system in which LacCer first binds to G_{M3} synthase (Kadowaki and Grant, 1994). UDP- and CMP-dialdehydes were demonstrated to be competitive inhibitors for embryonic chicken brain SAT-I (Cambron and Liskawa, 1993). The activity of SAT-I decreased ~2-fold in rat liver during the inflammatory response and, like ST6(N), the enzyme is released in serum in a soluble form after the cleavage of the N-terminal part of the molecule (Richardson and Jamieson, 1995).

The α2,8-sialyltransferase sub-family

*CMP-sialic acid: **NeuAcα2-3Galβ1-4GlcNAc-R** α2,8-sialyltransferase (EC 2.4.99.-; STX; # 14).* In mammals, α2-8-polysialic acid chains occur on embryonic and newborn N-CAM oligosaccharides, on the α subunit of voltage gated sodium channel in rat brain (Zuber *et al.*, 1992) and on the cell surface of numerous tumour cells (Troy, 1992). The poly (-8NeuAcα2-) linear structures are attached as outer branches to tri- and tetra-antennary N-linked oligosaccharide chains (Finne, 1982), linked to an α2-3-linked sialic acid residue. In fact, the biosynthesis of these polysialic acid chains would involve at least two distinct α2,8-sialyltransferases in mammals, as it is in *Escherichia coli* K1 (Troy, 1992) and in trout egg (Kitazume *et al.*, 1994): (i) the first one is an initiating enzyme that transfers a first sialic acid residue to the 8-hydroxyl group of NeuAc residue α2-3-linked to the terminal position of N-linked glycans (STX; #14); (ii) the second enzyme (poly-α2,8-ST; # 16) plays a role in the elongation of the α2-8-polysialic acid chains.

A recent cloning paper from Kojima *et al.* (1995) has reported that mouse STX (# 14) is probably the first initiating enzyme involved in this pathway. Indeed, several pieces of evidence indicate that the enzyme cloned from a newborn mouse brain cDNA library is able to transfer *in vitro* sialic acid in α2-8-linkage onto the α2-3- or α2-6-sialylated N-linked glycans of fetuin or α_1-acid glycoprotein, but is not able to sialylate asialoglycoproteins or gangliosides. Moreover, the mouse STX enzyme does not exhibit poly-α2,8-sialosyl α2,8-sialyltransferase activity.

Finally, the purification of a potential initiating enzyme from human neuroblastoma cells has been reported very recently by Stoykova and Glick (1995).

CMP-sialic acid: poly-α2,8-sialosyl α2,8-sialyltransferase (EC 2.4.99.-; poly-α2,8-ST; # 16). The poly-α2,8-ST enzyme catalyses the following reaction:

CMP-NeuAc + **NeuAcα2-8**(NeuAcα2-8)$_n$NeuAcα2-3-R → NeuAcα2-8(NeuAcα2-8)$_{n+1}$NeuAcα2-3-R + CMP

where R is one of the outer branches of a complex type tri- or tetra-antennary glycan (Finne, 1982) and $0 < n \leq 55$. The initial identification of a CMP-sialic acid: poly-α2,8-sialosyl α2,8-sialyltransferase in a eukaryotic organism was reported in fetal rat by McCoy *et al.* (1985). The enzyme appeared to be differentially expressed in developing neural tissues and its expression is apparently restricted to an early stage of development. Postnatal, but not adult rat brain, Golgi-enriched fractions catalyse the sialylation of N-CAM (Breen *et al.*, 1987). The characterization and purification of the α2,8-sialyltransferase from embryonic chick brain has been reported very recently by Sevigny *et al.* (1995) and also by Oka *et al.* (1995).

*CMP-sialic acid: **sialylα2-3Galβ1-4Glcβ1-O-Cer** α2,8-sialyltransferase (EC 2.4.99.8; SAT-II; G_{D3} synthase; # 17).* G_{D3} synthase (SAT-II in Figure 1) catalyses the following reaction:

CMP-sialic acid + **sialylα2-3**Galβ1-4Glcβ1-O-Cer → sialylα2-8**sialylα2-3**Galβ1-4Glcβ1-O-Cer + CMP

SAT-II has been characterized in embryonic chicken brain (Kaufman *et al.*, 1968), in rat liver (Pohlentz *et al.*, 1988; Mesaric and Decker, 1990) and purified to homogeneity from young rat brain (Gu *et al.*, 1990b). The only acceptor structure used by this synthase is G_{M3} (sialylα2-3Galβ1-4Glcβ1-O-Cer). Since the G_{D3} synthase does not utilize G_{M2} as an acceptor, G_{D2} must be synthesized from G_{D3} by the action of the β1-4-*N*-acetylgalactosaminyltransferase (Pohlentz *et al.*,1988). Thus, G_{D3} synthase strictly controls the *b*-pathway of ganglioside biosynthesis leading to G_{D3}, G_{D2}, G_{D1b}, G_{T1b} and G_{Q1b}. The increased activity of G_{D3} synthase has to be correlated with the high level of expression of G_{D3} in some tumour cells, such as melanoma cells (Furokawa *et al.*, 1989). Recently, SAT-II cDNA has been cloned by three different groups (Haraguchi *et al.*, 1994; Nara *et al.*, 1994; Sasaki *et al.*, 1994).

*CMP-sialic acid: **sialylα2-8sialylα2-3Galβ1-4Glcβ1-O-Cer** α2,8-sialyltransferase (EC 2.4.99.-; SAT-III; G_{T3} synthase; # 18).* The putative G_{T3} synthase, represented in Figure 1 as SAT-III, catalyses the following reaction:

CMP-sialic acid + **sialylα2-8**sialylα2-3Galβ1-4Glcβ1-O-Cer → sialylα2-8**sialylα2-8**sialylα2-3Galβ1-4Glcβ1-O-Cer + CMP

However, SAT-III has not yet been characterized or purified in mammalian tissues or cells and, according to *in vitro* assay results, the synthesis of G_{T3} could be catalysed by SAT-V in rat liver (Iber *et al.*, 1992). Surprisingly, a very recent report on the expression cloning of the human G_{T3} synthase (SAT-III) indicated that the enzyme was identical to the G_{D3} synthase (SAT-II) (Nakayama *et al.*, 1995).

*CMP-sialic acid: **NeuAcα2-3Galβ1-3GalNAcβ1-4[R-]Galβ1-4Glcβ1-O-Cer** α2,3-sialyltransferase (EC 2.4.99.-; SAT-V; # 19).* SAT-V catalyses the following reaction:

CMP-sialic acid + **sialylα2-3Galβ1**-3GalNAcβ1-4[R-]Galβ1-4Glcβ1-O-Cer → sialylα2-8**sialylα2-3Galβ1**-3GalNAcβ1-4[R-]Galβ1-4Glcβ1-O-Cer + CMP

where R = H (acceptor substrate G_{M1b}), or sialylα2-3 (G_{D1a}), or sialylα2-8sialylα2-3 (G_{T1b}), or sialylα2-8sialylα2-8sialylα2-3 (G_{Q1c}) (see Figure 1). SAT-V has been characterized in rat liver (Iber and Sandhoff, 1989; Iber *et al.*, 1992). In addition to the natural precursors used as acceptor substrates (G_{M1b}, G_{D1a}, G_{T1b}, G_{Q1c}), it has been demonstrated by competition assays between G_{D3} and G_{D1a} that the rat liver SAT-V is also able to transfer sialic acid to the 8-hydroxyl group of the terminal sialic acid residue of G_{D3} (**sialylα2-8**sialylα2-3Galβ1-4Glcβ1-O-Cer) in an *in vitro* assay (Iber *et al.*, 1992).

Cloning of sialyltransferase cDNAs

The data summarized in Tables IV and V show the dramatic advances that have been made during this past year in the cloning of sialyltransferase cDNAs. The sialyltransferases for which cDNAs have been obtained and that are required for the synthesis of the known sialyl-oligosaccharide structures (Table II) are listed in Table IV. As discussed above, since glycosyltransferases are generally non-abundant proteins, it has been very difficult to purify these enzymes for molecular cloning purposes. Furthermore, although sialyltransferases are related by function, there was little information available to evaluate the degree to which these enzymes were structurally related. However, this classical purification procedure was chosen to clone the cDNA of two mammalian

Table IV. Cloned mammalian and avian sialyltransferase cDNAs. The bold character sugar residue is the transferred sialic acid. C = cytoplasmic; TM = transmembrane domain; L = luminal domain

Number[a]	Structure	Species	Domains C/TM/L	References	Accession number
1	NeuAcα2-6Galβ1-4GlcNacβ-	rat liver	7/17/377	Weinstein *et al* (1987)	M18769
		human placenta	9/17/380	Grundman *et al.* (1990)	X17247
		mouse liver		Hamamoto *et al.* (1993)	D16106
		chicken embryo	9/17/387	Kurosawa *et al* (1994b)	X75558
3	(NeuAcα2-3)$_{0-1}$(Galβ1-3)$_{0-1}$GalNAc-Ser / NeuAcα2-6	chicken embryo	17/21/528	Kurosawa *et al.* (1994a)	X74946
4	(NeuAcα2-3)$_{0-1}$Galβ1-3GalNAc-Ser / NeuAcα2-6	chicken testis	8/17/379	Kurosawa *et al* (1994c)	X77775
5	NeuAcα2-3Galβ1-3GalNAc-Ser / NeuAcα2-6	rat brain		Sjoberg *et al.* (1995)	
6	NeuAcα2-3Galβ1-3/4GalNAcβ-	rat liver	8/20/346	Wen *et al* (1992a)	M97754
		human placenta	8/20/347	Kitagawa and Paulson (1993)	JN0618
7	NeuAcα2-3Galβ1-3/4GalNAcβ-SLe^x	human melanoma	8/18/303	Sasaki *et al.* (1993)	X74570
9	NeuAcα2-3Galβ1-3GalNAc-	porcine submaxillary gland	11/16/316	Gillepsie *et al.* (1992)	M97753
		mouse brain	7/16/314	Lee *et al.* (1993)	X73523
		human placenta	10/16/314	Kitagawa and Paulson (1994b)	L29555
		chicken embryo brain	10/18/314	Kurosawa *et al.* (1995)	X8053
10	NeuAcα2-3Galβ1-3GalNAc	mouse brain	6/21/323	Lee *et al.* (1994)	X76988
		rat brain	6/21/323	Lee *et al.* (1994)	X76989
11	NeuAcα2-3Galβ1-3GalNAc NeuAcα2-3Galβ1-4GlcNAc[b]	human placenta	7/18/307	Kitagawa and Paulson (1994a)	L23767
14	NeuAcα2-8NeuAcα2-3Galβ1-4GlcNAc	rat brain (newborn)	6/17/352	Livingston and Paulson (1993)	L13445
		human placenta		Kitagawa and Paulson (1994)a	L29556
		mouse fetal brain		Kojima *et al.* (1995)	
15	NeuAcα2-8NeuAcα2-3Galβ1-4GlcNAc	mouse brain	17/16/357	Yoshida *et al.* (1995a)	X80502
16	NeuAcα2-8(NeuAcα2-8)$_n$NeuAcα2-3Galβ1-R	CHO cells	7/13/339	Eckhardt *et al.* (1995)	Z46801
		human fetal brain	7/13/339	Nakayama *et al.* (1995)	L41680
17	NeuAcα2-8NeuAcα2-3Galβ1-4Glc-Cer G_{D3}	human melanoma	12/22/277	Nara *et al.* (1994)	D26360
		human melanoma	29/19/308	Sasaki *et al.* (1994)	X77922
		human melanoma	14/18/309	Haragushi *et al.* (1994)	L32867

[a]The numbers of sialyltransferases correspond to those in Table I.
[b]Refers to the preferred acceptor.

α2,3-sialyltransferases; the proteins have been purified from several sources, their amino acid sequence partially analysed, leading to their molecular cloning.

Molecular biology is now beginning to provide enough cDNA probes to address fundamental questions such as the targeting of the sialyltransferases to the Golgi apparatus. The following sections review the different approaches followed and the results obtained for the cloning of sialyltransferase cDNAs.

Screening of a cDNA library using oligonucleotides probe derived from amino acid sequence of the purified enzyme

Two forms of the CMP-sialic acid: Galβ1-3GalNAc-R α2,3-sialyltransferase (ST3(O)-I; # 9) of mol. wt 45 and 48 kDa were purified from porcine liver extracts (Gillespie *et al.*, 1992). A 53 bp non-degenerate oligonucleotide probe corresponding to the amino terminal sequence of the 48 kDa enzyme was used to screen a λgt10 cDNA library from porcine submaxillary glands, and two overlapping cDNA clones named ST1 and ST2 were purified and characterized. Expression of a soluble and active enzyme was obtained by transient transfection of COS-1 cells with the recombinant cDNA ST2. This enzyme has been shown to exhibit activity toward the Galβ1-3GalNAc structure found on both glycoproteins and glycolipids.

The CMP-sialic acid: Galβ1-3(4)GlcNAc-R α2,3-sialyltransferase (ST3(N), # 6) was purified from rat liver (Weinstein *et al.*, 1982a). The protein was reduced, alkylated, tryptic digests were carried out and the resulting peptides were fractionated by HPLC. Mass spectrometry was used for a rapid structural elucidation of the amino acid sequence of the enzyme. Based on the amino acid sequences of 11 of the 14 peptides analysed, 22 degenerate sense and antisense oligonucleotides were synthesized to carry out PCR on rat liver cDNA and a nearly full-length cDNA clone was isolated (Wen *et al.*, 1992a). In order to define the enzymatic properties of the enzyme, a soluble form of the sialyltransferase was constructed in the mammalian expression vector pSVL and transient expression was achieved in COS-1 cells.

Screening of a bacterial expression library with an antibody probe

The first sialyltransferase cloned was the CMP-sialic acid: Galβ1-4GlcNAc-R α2,6-sialyltransferase (ST6(N), # 1; Weinstein *et al.*, 1987). Because polyclonal antibodies to the purified sialyltransferase had been raised previously (Weinstein *et al.*, 1982a), an expression λgt11 bacterial library prepared from rat liver mRNA was screened. A single positive clone producing a

Table V. Cloning strategies for the isolation of sialyltransferase cDNAs

Number[a]	Species	References	Vector used	Cloning based on
1	rat liver	Weinstein *et al.* (1987)	λgt11	Antibody
	human placenta	Grundman *et al.* (1990)	λgt10	Homology screening
	mouse liver	Hamamoto *et al.* (1993)	–	Single-primer PCR
	chicken embryo	Kurosawa *et al.* (1994b)	pcDS-A2	Sialylmotif nucleotide sequence/PCR
3	chicken embryo	Kurosawa *et al.* (1994a)	λZAPII	Sialylmotif nucleotide sequence/PCR
4	chicken testis	Kurosawa *et al.* (1994c)	λZAPII	Sialylmotif nucleotide sequence/PCR
5	rat brain	Sjoberg *et al.* (1995)	–	Sialylmotif nucleotide sequence/PCR
6	rat liver	Wen *et al.* (1992a)	λgt10	NH_2 terminal sequence/PCR
	human placenta	Kitagawa and Paulson (1993)	λZAPII	Sialylmotif nucleotide sequence/PCR
7	human melanoma	Sasaki *et al.* (1993)	pAMo	Expression cloning, selection lectin RCA
9	porcine submaxillary gland	Gillepsie *et al.* (1992)	λgt10	NH_2 terminal sequence/PCR
	mouse brain	Lee *et al.* (1993)	Uni-ZAPXR	Sialylmotif nucleotide sequence/PCR
	human placenta	Kitagawa and Paulson (1994b)	λZAPII	Sialylmotif nucleotide sequence/PCR
10	mouse brain	Lee *et al.* (1994)	λZAPII	Sialylmotif nucleotide sequence/PCR
	rat brain	Lee *et al.* (1994)	λZAPII	Sialylmotif nucleotide sequence/PCR
11	human placenta	Kitagawa and Paulson (1994a)	λZAPII	Sialylmotif nucleotide sequence/PCR
14	rat brain (newborn)	Livingston and Paulson (1993)	λgt10	Sialylmotif nucleotide sequence/PCR
	human placenta	Kitagawa and Paulson (1994)	λZAPII	Sialylmotif nucleotide sequence/PCR
	mouse fetal brain	Kojima *et al.* (1995)	λZAPII	Sialylmotif nucleotide sequence/PCR
15	mouse brain	Yoshida *et al.* (1995)	pcDSA	Sialylmotif nucleotide sequence/PCR
16	CHO cells	Eckhardt *et al.* (1995)	pcDM8	Expression cloning, selection anti-PSA mAb 735
	human fetal brain	Nakayama *et al.* (1995)	pcDM8	Expression cloning, selection anti-PSA mAb 735
17	human melanoma	Nara *et al.* (1994)	pCEV18	Expression cloning, selection anti-GD3 mAb R24
	human melanoma	Sasaki *et al.* (1994)	pAMo	Expression cloning, selection anti-GD3 mAb KM641
	human melanoma	Haragushi *et al.* (1994)	pcDM8	Expression cloning, selection anti-GD2 mAb in transfected cells

[a]The numbers of sialyltransferases correspond to those in Table I.

β-galactosidase–sialyltransferase fusion protein was identified and purified (Weinstein *et al.*, 1987).

Homology screening based on previously isolated clones

A cDNA sequence of the human CMP-sialic acid: Galβ1-4-GlcNAc-R α2,6-sialyltransferase (ST6(N), # 1) was obtained by Grundman *et al.* (1990) from a human placental λgt10 library. Two oligonucleotides generated from the rat liver sialyltransferase previously cloned were used to screen the human library and to isolate a positive clone. The corresponding protein sequence showed 87.6% similarity to the rat protein sequence.

The same strategy was used by Lance *et al.* (1989) to isolate and characterize a partial human cDNA of the CMP-sialic acid: Galβ1-4GlcNAc-R α2,6-sialyltransferase (ST6(N), # 1) from a human submaxillary gland λgt11 library. At the amino acid level, 86% conservation between this human clone and the rat α2,6-sialyltransferase sequence was observed.

Expression cloning system, transfection of cDNA library into CHO or COS cells

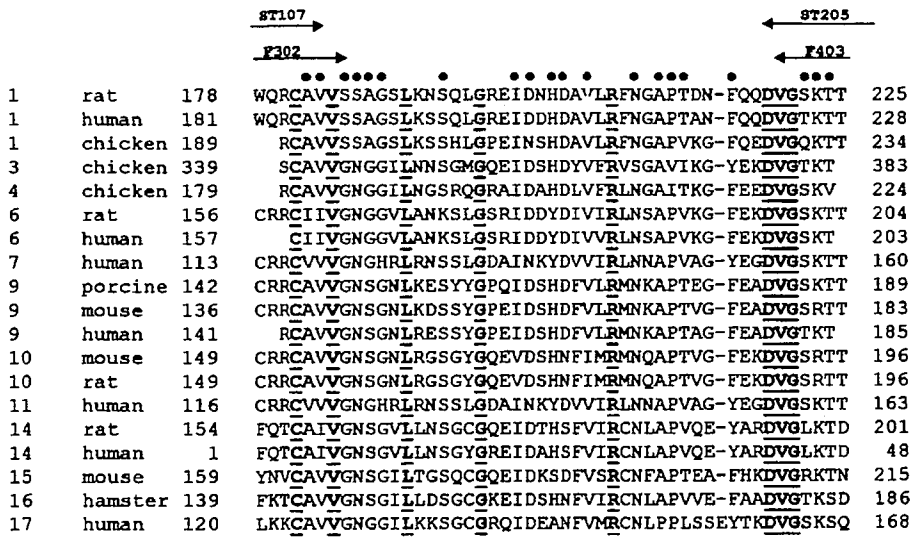
Sasaki *et al.* (1993) recently reported the use of an expression cloning system to isolate the human CMP-sialic acid: Galβ1-3(4)GlcNAc-R α2,3-sialyltransferase (# 7) cDNA that directs *de novo* expression of the SLe^x determinant.

The human Burkitt lymphoma cell line Namalwa (a B-cell line) was stably transfected with a cDNA library prepared from WM266-4 cells, in the expression vector pAMo, an Epstein–Barr virus-based cloning vector. One single plasmid DNA was identified that conferred the RCA_{120} (*Ricinus communis* agglutinin 120) resistance phenotype. This cytotoxic lectin RCA_{120} was used because it was predicted that sialylation of the Galβ1-4GlcNAc structure (recognized by RCA_{120}) would increase the level of lectin resistance of these Namalwa cells, which otherwise were found to express the SLe^x antigen poorly.

The same group (Sasaki *et al.*, 1994) used the same strategy to clone the G_{M3}-specific α2,8-sialyltransferase or G_{D3} synthase (# 17) a year later. Namalwa cells were found to contain abundant G_{M3}, an acceptor substrate for G_{D3} synthase, whereas both G_{D3} and G_{D3} synthase were not expressed. Namalwa cells stably transfected with a human melanoma cDNA library were subjected to sorting on a flow cytometer with anti-G_{D3} monoclonal antibody (mAb) KM643, and the individual plasmids rescued from the selected cells were analysed.

Expression cloning of the same G_{M3}-specific α2,8-sialyltransferase (# 17) was achieved by changing the selection strategy, selecting G_{D2} instead of G_{D3} with an anti-G_{D2} mAb (Haragushi *et al.*, 1994). A host recipient cell line (KF 3027 Hyg5) expressing high levels of G_{M2} and G_{M3}, but not G_{D2} or G_{D3} (no G_{D3} synthase activity), was obtained after transfection of mouse B16 melanoma cells with the polyoma large tumour antigen (LT) and the previously cloned β1,4-*N*-acetylgalactosaminyltransferase cDNA. Two cDNA clones have been isolated and transfected in KF3027-Hyg5 cells where they direct the expression of G_{D3} and G_{D2}.

Sialylmotif L



1	rat	178	WQRCAVVSSAGSLKNSQLGREIDNHDAVLRFNGAPTDN-FQQDVGSKTT	225
1	human	181	WQRCAVVSSAGSLKSSQLGREIDDHDAVLRFNGAPTAN-FQQDVGTKTT	228
1	chicken	189	RCAVVSSAGSLKSSHLGPEINSHDAVLRFNGAPVKG-FQEDVGQKTT	234
3	chicken	339	SCAVVGNGGILNNSGMGQEIDSHDYVFRVSGAVIKG-YEKDVGTKT	383
4	chicken	179	RCAVVGNGGILNGSRQGRAIDAHDLVFRLNGAITKG-FEEDVGSKV	224
6	rat	156	CRRCIIVGNGGVLANKSLGSRIDDYDIVIRLNSAPVKG-FEKDVGSKTT	204
6	human	157	CIIVGNGGVLANKSLGSRIDDYDIVVRLNSAPVKG-FEKDVGSKT	203
7	human	113	CRRCVVVGNGHRLRNSSLGDAINKYDVVIRLNNAPVAG-YEGDVGSKTT	160
9	porcine	142	CRRCAVVGNSGNLKESYYGPQIDSHDFVLRMNKAPTEG-FEADVGSKTT	189
9	mouse	136	CRRCAVVGNSGNLKDSSYGPEIDSHDFVLRMNKAPTVG-FEADVGSRTT	183
9	human	141	RCAVVGNSGNLRESSYGPEIDSHDFVLRMNKAPTAG-FEADVGTKT	185
10	mouse	149	CRRCAVVGNSGNLRGSGYGQEVDSHNFIMRMNQAPTVG-FEKDVGSRTT	196
10	rat	149	CRRCAVVGNSGNLRGSGYGQEVDSHNFIMRMNQAPTVG-FEKDVGSRTT	196
11	human	116	CRRCVVVGNGHRLRNSSLGDAINKYDVVIRLNNAPVAG-YEGDVGSKTT	163
14	rat	154	FQTCAIVGNSGVLLNSGCGQEIDTHSFVIRCNLAPVQE-YARDVGLKTD	201
14	human	1	FQTCAIVGNSGVLLNSGYGREIDAHSFVIRCNLAPVQE-YARDVGLKTD	48
15	mouse	159	YNVCAVVGNSGILTGSQCGQEIDKSDFVSRCNFAPTEA-FHKDVGRKTN	215
16	hamster	139	FKTCAVVGNSGILLDSGCGKEIDSHNFVIRCNLAPVVE-FAADVGTKSD	186
17	human	120	LKKCAVVGNGGILKKSGCGRQIDEANFVMRCNLPPLSSEYTKDVGSKSQ	168

Sialylmotif S

1	rat	318	PSSGMLGIIIMMTLCDQVDIYEF	340
1	human	321	PSSGMLGIIIMMTLCDQVDIYEF	343
1	chicken	328	PSSGMLGIVIMMTLCDEVDVTEF	350
3	chicken	494	PTTGALLLLTALHLCDRVSAYGY	516
4	chicken	332	PSTGALMLLTALHTCDQVSAYGF	354
6	rat	299	PTLGSVAVTMALDGCDEVAVAGF	321
6	human	300	PTLGSVAVTMALHGCDEVAVAGF	322
7	human	255	PTTGLLAITLALHLCDLVHIAGF	277
9	porcine	270	PSTGILSVIFSLHICDEVDLYGF	292
9	mouse	264	PSTGILSIIFSIHICDEVDLYGF	286
9	human	267	PSTGILSVIFSMHVCDEVDLYGF	289
10	mouse	277	PSTGMLVLFFALHVCDEVNVYGF	299
10	rat	277	PSTGMLVLFFALHVCPEVNVTGF	299
11	human	258	PTTGLLAITLALHLCDLVHIAGF	280
14	rat	293	PTTGLLMYTLATRFCNQIYLYGF	315
14	human	140	PTTGLLMYTLATRFCKQIYLYGF	162
15	mouse	299	LSTGILMYTLASAICEEIHLYGF	321
16	hamster	278	PSTGLLMYTLATRFCDEIHLYGF	300
17	human	258	LSTGLFLVSAALGLCEEVAIYGF	280

Fig. 2. Comparison of the deduced amino acid sequences in the two conserved regions of the sialyltransferase gene family named sialylmotif L and sialylmotif S, respectively. Alignment of the amino acid conserved regions was conducted for the 12 sialyltransferase sequences and for each species cloned. Underlined and bold-face letters indicate a perfectly conserved position in the alignment, the dots indicate a position that is well conserved. Numbers on the left side of the figure refer to those in Table I.

Finally, Nara *et al.* (1994) used a transient expression cloning system in polyoma LT antigen-expressing Chinese hamster ovary (CHO) cells to clone the cDNA of the G_{M3}-specific α2,8-sialyltransferase (# 17). G_{D3} synthase was isolated from a cDNA library prepared from the mRNA of the SK-Mel-28 melanoma cell line and transfected CHO cells were selected using a fluorescence activated cell sorting method.

Very recently, Eckhardt *et al.* (1995) have reported the expression cloning and molecular characterization of eukaryotic polysialyltransferase-1 (PST-1, # 16). A mutant clone of CHO cells shown to be defective in polysialic acid (PSA) by negative selection on the PSA-specific mAb 735 was used for transfection with a complementary DNA library from CHO-WT cells in pcDM8 vector. PSA-positive cells were collected by panning on antibody 735, and plasmid DNA was subsequently extracted and analysed.

PCR methods based on nucleotide sequence

On the basis of the data obtained from the cloning of the first three sialyltransferases (# 1, Weinstein *et al.*, 1987; # 6, Wen *et al.*, 1992a; # 9, Gillepsie *et al.*, 1992), analysis of the deduced protein sequence has revealed that sialyltransferases contained two conserved regions called the 'sialylmotif' (Figure 2) (Drickamer, 1993; Livingston and Paulson, 1993). One region is a 45 amino acid stretch found roughly in the

centre of the coding sequence (sialylmotif L). It is conserved among all sialyltransferases cloned to date. The other region is a small stretch of 23 amino acids at the carboxyl-terminal end of the molecule (sialylmotif S). None of the residues in the conserved motif are found in other glycosyltransferases.

What is the function of the two highly conserved regions, sialylmotif L and S? Datta and Paulson (1995) have clarified this function by site-directed mutagenesis, changing selected conserved amino acids in the larger conserved domain to alanine, and have shown that this motif is involved in the binding of CMP-NeuAc. The conservation of the sialylmotif in all three cloned sialyltransferases predicted that other members of the sialyltransferase gene family might also contain the same motif and thus could be used to isolate new sialyltransferase clones and, indeed, PCR-based approaches using sequence information in the sialylmotif have provided an attractive alternative for obtaining new members of the sialyltransferase gene family (see Tables III–V and Figure 2).

Three CMP-sialic acid: α2,6-sialyltransferases were cloned from chick embryonic cDNA libraries using sequence information obtained from the conserved amino acid sequence (the so-called sialylmotif L) of the previously cloned mammalian sialyltransferases. PCR was performed using the degenerate 5′ primer ST107 and 3′ primer ST205 (indicated in Figure 2) defined from this conserved region, and predicted to generate a fragment of 150 bp. This fragment was further used to screen a chick embryo cDNA library.

(i) The CMP-sialic acid: Galβ1-4GlcNAc α2,6-sialyltransferase (ST6(N); # 1) cDNA sequence obtained reveals an open reading frame encoding 413 amino acids that shows 57.6% identity with the rat liver enzyme. Expression of the recombinant protein in COS-7 cells resulted in the secretion of a catalytically active and soluble form of the enzyme (Kurosawa *et al.*, 1994b).

(ii) The cDNA sequence of CMP-sialic acid: R-GalNAcα1-Ser α2,6-sialyltransferase, in which R = H or Galβ1-3 or NeuAcα2-3Galβ1-3 (ST6GalNAc-I; # 3), reveals an open reading frame encoding 566 amino acids that shows 12% identity with that of the previous enzyme (# 1, from chick embryo). Expression and identification of the enzyme activity were achieved in COS-7 cells (Kurosawa *et al.*, 1994a).

(iii) The cDNA sequence of the CMP-sialic acid: Galβ1-3GalNAcα1-Ser α2,6-sialyltransferase enzyme (ST6GalNAc-II, # 4) predicts amino acid sequence with 32% identity to that of ST6NGalNAc-I (# 3). A cDNA clone encoding the full-length cDNA was expressed in COS-7 cells and the enzyme activity identified (Kurosawa *et al.*, 1994c). This enzyme differs though from the previously purified CMP-sialic acid: NeuAcα2-3Galβ1-3GalNAcα1-Ser α2,6-sialyltransferase (EC 2.4.99.7; # 5) which has a strict requirement for the trisaccharide NeuAcα2-3Galβ1-3GalNAcα1-R as an acceptor substrate.

Four CMP-sialic acid: α2,3-sialyltransferases were cloned from human placental libraries in λZAPII using degenerate 5′ and 3′ primers corresponding to conserved regions at the ends of the sialylmotif L (not represented in Figure 2) predicted to yield a 150 bp amplified fragment: (i) the human CMP-sialic acid: Galβ1-3GalNAc-R α2,3-sialyltransferase (ST3(O)-I; # 9; Kitagawa and Paulson, 1994b); (ii) the human CMP-sialic acid: Galβ1-4GlcNAc-R or Galβ1-3GalNAc-R α2,3-sialyltransferase (ST3(N/O); # 11; Kitagawa and Paulson, 1994a); (iii) the human CMP-sialic acid: Galβ-3GlcNAc-R α2,3-sialyltransferase (ST3(N); # 6; Kitagawa and Paulson,1993); (iv) the human CMP-sialic acid NeuAcα2-3Galβ1-4GlcNAc-R α2,8-sialyltransferase (STX; # 14; Kitagawa and Paulson, 1994a). Using another set of primers derived from the sequence information of the 150 bp amplified fragment, screening of a human fetal brain cDNA library yielded a partial cDNA clone (620 bp) of human STX with unknown sialyltransferase activity. Comparison of the deduced amino acid sequence from the partial human cDNA with the rat cDNA previously cloned (Livingston and Paulson, 1993) shows the highest conservation observed to date between sialyltransferase genes. Very recently, Kojima *et al.* (1995) cloned the mouse STX gene from mouse fetal brain and expressed a truncated form of STX in COS-7 cells. They found that mouse STX is a novel α2,8-sialyltransferase highly regulated during brain development and specific for sialylated (both α2,3- and α2,6-linked sialic acid) N-linked oligosaccharides of glycoproteins.

Two other CMP-sialic acid: Galβ1-3GalNAc α2,3-sialyltransferases (# 10) from mouse brain and rat brain cDNA libraries were cloned using sense primer F302 and antisense primer F403 that corresponded to the conserved regions of the sialylmotif L of the previously cloned sialyltransferases (represented in Figure 2). (i) The mouse CMP-sialic acid: Galβ1-3GalNAc α2,3-sialyltransferase (ST3GalA.1; # 9; Lee *et al.*, 1993). The identity of this enzyme was confirmed by construction of a recombinant sialyltransferase and expression in COS-7 cells, and it corresponds to the enzyme originally cloned in porcine submaxillary glands (Gillepsie *et al.*, 1992). (ii) The mouse CMP-sialic acid: Galβ1-3GalNAc α2,3-sialyltransferase (ST3GalA.2; # 10). Lee *et al.* (1994) reported the cloning of another type of α2,3-sialyltransferase from mouse brain and rat brain that had similar substrate specificities to mouse ST3GalA.1 (# 9), but exhibited striking acceptor substrate preferences. Asialo-GM_1 and GM_1 were much more suitable substrates for ST3GalA.2 than for the ST3GalA.1 (Kojima *et al.*, 1994), leading the authors to conclude that ST3GalA.1 may be related to glycoprotein biosynthesis, whereas ST3GalA.2 would be related to glycolipid biosynthesis. Northern blot analysis from several mouse tissues also showed a different tissue distribution with ST3GalA.1 mRNAs being expressed mainly in submaxillary glands, to a lesser extent in liver and heart, but not in brain, lung and kidney. ST3GalA.2 mRNAs are highly expressed in brain and liver, to a lesser extent in heart and kidney, but not in submaxillary glands, spleen and pancreas.

Very recently, a cDNA encoding a new α2,8-sialyltransferase (# 15) was cloned from mouse brain by means of the PCR-based method (Yoshida *et al.*, 1995a). Degenerated primers (not represented in Figure 2), based on the conserved sequences of the sialylmotif L found in rat brain STX and in human melanoma GD_3 synthase, were used to generate a 500 bp PCR fragment subsequently used to screen a 3-day-old mouse brain cDNA library. Functional analysis of the new sialyltransferase was conducted by transfection of the expression plasmid pcDSA 03 in COS 7 cells, which led to activity preferentially toward sialylated glycoproteins, but also toward α2,3-sialylated glycosphingolipids.

Hamamoto *et al.* (1993) developed an original two-step single primer-mediated PCR to clone a putative mouse CMP-sialic acid: Galβ1-4GlcNAc α2,6-sialyltransferase cDNA. Mouse cDNA was used as a template to generate a 150 bp fragment with degenerate PCR primers corresponding to the 'sialylmotif L'. This fragment was further subcloned into a plasmid vector and two PCRs were performed with single primer for both the 5′ and 3′ sides, giving rise to a

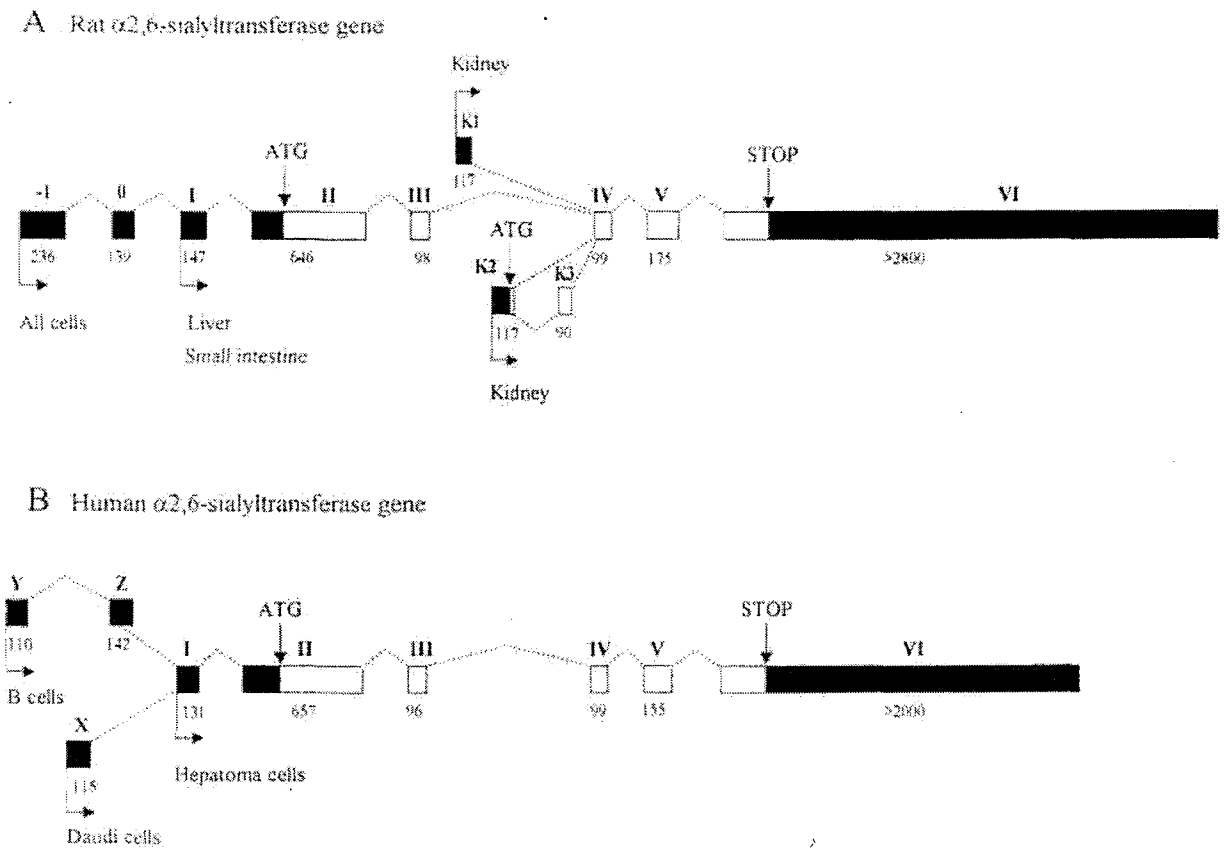


Fig. 3. Genomic map of (A) rat CMP-sialic acid: Galβ1-4GlcNAc-R α2,6-sialyltransferase and (B) human CMP-sialic acid: Galβ1-4GlcNAc-R α2,6-sialyltransferase. The numbered and lettered boxes indicate the exons. The black boxes represent the untranslated sequences, whereas open boxes indicate the open reading frame. The downward bent arrows indicate the transcriptional start site, and the positions of ATG and stop codons are indicated by an arrow. The number underneath each box is the length of the exon in base pairs.

2.2 kb sequence containing the full coding region of mouse β-galactoside α2,6-sialyltransferase.

Cloned cDNAs for several sialyltransferases have now become available, permitting molecular approaches towards the understanding of their biosynthesis, their specific subcellular location and even the structural basis for their substrate specificities. They also provide excellent tools for the analysis of sialyltransferase gene expression and the next section summarizes the recent insights that we have gained.

Regulation of spatio-temporal expression of sialyltransferase genes

Variations in complex carbohydrate structures have been observed during development, differentiation, in disease processes and between different normal tissues, leading to the conclusion that the expression of terminal sequences is strictly controlled both spatially and temporally (Feizi, 1985). Glycosyltransferase spatio-temporal expression appears to be regulated mainly at the level of transcription, although additional controls on enzyme activity may be exerted post-transcriptionally by differential processing of the mRNA, by altering the stability of the mRNA and/or by differences in the translational efficiency. In this context, it has to be noted that the extraordinarily long 5′ and 3′ untranslated regions of the glycosyltransferase transcripts allow potential secondary structures to form and regulatory proteins to bind.

Similar to housekeeping genes, glycosyltransferase genes appear to be constitutively expressed at relatively low level in most mammalian somatic cells. A basal level of transcription ensures glycosylation of cellular proteins and lipids and, in addition to this basal level of expression, there may be a cell type-specific and/or time- and/or differentiation-dependent regulation in transcription rate, for example through hormonal pathways.

Regulation of the expression of sialyltransferase genes is just beginning to be studied and published data concern essentially rat and human Galβ1-4GlcNAc-R α2,6-sialyltransferase (ST6 (N), #1) and human Galβ1-3GalNAc α2,3-sialyltransferase (# 9).

Northern blot analysis of rat tissue mRNAs using rat liver ST6(N) (#1) cDNA as a probe (Weinstein *et al.*, 1987) has identified at least five transcripts with distinct size and expression patterns (O'Hanlon *et al.*, 1989; Paulson *et al.*, 1989; Wen *et al.*, 1992b). The rat ST6(N) gene is surprisingly complex with 11 exons spanning over 80 kb and at least four promoters that regulate its expression (Figure 3A).

In most rat tissues, transcription initiates from an upstream promoter not yet defined, that drives the low-level constitutive expression of a 4.7 kb transcript (Wen *et al.*, 1992). Vandamme *et al.* (1993) have described an upregulation in the specific expression of this 4.7 kb mRNA in rat fibroblasts (FR3T3 cells)

cultured in the presence of dexamethasone directly dependent on the glucocorticoid receptor pathway. Le Marer *et al.* (1992) have also observed a striking increase in the expression of the 4.7 kb mRNA upon *ras* transformation of rat fibroblasts FR3T3, leading to a high α2,6-sialylation of *N*-acetyllactosamine sequences which correlates with the high invasive potential of these cells (Le Marer and Stéhelin, 1995). Finally, Grollman *et al.* (1993) have presented evidence that thyroid-stimulating hormone (TSH) negatively regulates a 4.7 kb mRNA species of CMP-sialic acid: α2,6-sialyltransferase in rat thyroid FRTL-5 cells. The promoter governing the expression of the 4.7 kb mRNA species remains to be characterized and the mechanism by which *ras* or dexamethasone induces its specific overexpression remains to be determined.

A downstream promoter containing consensus binding sites for several liver restricted transcription factors HNF-1 (hepatocyte nuclear factor 1α), DBP (D-binding protein) and LAP (liver-enriched transcriptional activator protein) allows the very high level of expression of this gene in the liver (Svensson *et al.*, 1990), giving rise to a 4.3 kb transcript lacking two exons in the 5′ untranslated region. Superimposed on this tissue-specific regulation of α2,6-sialyltransferase gene expression, induction of sialyltransferase activity and enrichment of the 4.3 kb mRNA by dexamethasone have been reported (O'Hanlon *et al.*, 1989; Wang *et al.*, 1989, 1990b). It is interesting to note the absence of the consensus motif corresponding to the glucocortocoid response element (GRE) (Evans, 1988; Beato, 1989) within 1 kb upstream of the transcriptional start site (Wang *et al.*, 1990b). Recently, it has been shown that the α2,6-sialyltransferase gene is strongly expressed in the newborn rat small intestinal mucosa and that its expression is strikingly downregulated during the weaning period. Furthermore, the results obtained show the presence in the small intestine of a 4.3 kb transcript identical to the hepatic form which would originate from the same promoter region residing immediately upstream of the hepatic transcriptional start site (Wang *et al.*, 1990a,b; Vertino-Bell *et al.*, 1994).

In the rat, two alternative promoters would generate three short kidney-specific transcripts of 3.6 kb. These three transcripts originate within an intron located between exons III and IV, and yield spliced products (Figure 3A). Although two of these three transcripts code for potentially truncated proteins, it remains to be determined whether these proteins are actually synthesized by kidney cells and are functional (Wen *et al.*, 1992).

A partial human cDNA sequence for the α2,6-sialyltransferase (# 1) was isolated at first from submaxillary gland (Lance *et al.*, 1989) and putative full-length cDNAs have been isolated from human placenta STP (Grundman *et al.*, 1990) and Daudi cells STB (Stamenkovic *et al.*, 1990) which are Burkitt lymphoma B-lymphoblastoid cells. These two cDNAs have identical coding regions and differ only in part of their 5′ untranslated region. Figure 3B represents the human α2,6-sialyltransferase genomic DNA which is very similar in its organization to the rat α2,6-ST gene. Human exons E(Y) and E(Z) present in STP are the homologue of the rat isotype exons E(−1) and E(0), whereas human exon E(X) has no counterpart, as yet, in the rat. In addition, results have been obtained to indicate the existence of a third α2,6-sialyltransferase transcript in human hepatocarcinoma cells ($HepG_2$ cells) lacking both exons E(Y) and E(Z) or E(X) (Aasheim *et al.*, 1993; Wang *et al.*, 1993), leading the authors to conclude that at least three physically distinct promoter regions are operative in the

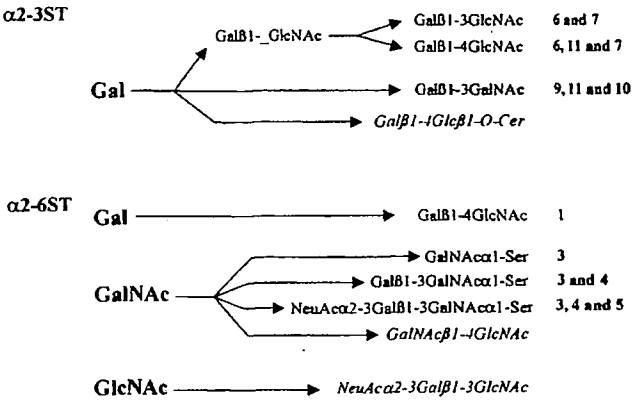


Fig. 4. Disaccharide sequences used by α2,3-ST and α2,6-ST as acceptor and the corresponding cloned enzymes. The sugar in bold characters is the acceptor sugar residue. The numbers on the right side of the figure refer to the cloned sialyltransferases listed in Table I. The acceptor structures in italic characters are those for which there are no cloned enzymes.

expression of the human α2,6-sialyltransferase (Figure 3B) (Aasheim *et al.*, 1995).

Concerning the human Galβ1-3GalNAc α2,3-sialyltransferase (ST3(O)-I, # 9), Northern analysis using mRNAs from human adult tissues and the human cDNA of the ST3(O)-I probe shows a major mRNA species of ~6.0 kb, while several smaller sized mRNAs of minor importance are also detected (Kitagawa and Paulson, 1994b).

Gillepsie *et al.* (1993) have presented evidence for the regulated expression of the CMP-sialic acid: α2,3-sialyltransferase gene (ST3(O)-I, # 9) in developing thymocytes. Cortical regions of the thymus composed of immature thymocytes show low levels of the 5.7 kb mRNA, while medullary regions of the thymus composed of mature thymocytes overexpress the 5.7 kb mRNA.

Summary and future prospects

During this past year, the availability of highly defined analytical tools such as PCR technology and information concerning previously cloned sialyltransferases has resulted in a vast increase in the number of cloned sialyltransferases. These data are summarized in Tables III and IV, and Figure 4, and it is possible to underline several interesting points.

Obviously, not all mammalian sialyltransferases have been cloned to date. This is the case, for example, for the CMP-sialic acid: NeuAcα2-3Galβ1-3GlcNAc α2,6-sialyltransferase (# 2) which was characterized in rat liver (Paulson *et al.*, 1984; de Heij *et al.*, 1986) or for G_{M3} synthase (# 12). This is also the case for ST6GalNAc-I (# 3) and ST6GalNAc-II (# 4) which have not yet been cloned from mammalian sources. However, Sjoberg *et al.* (1995) reported the cDNA cloning and expression of ST6(O)-II (ST6GalNAc-III, # 5) from a rat brain cDNA library. Although the avian cloned former enzyme ST6GalNAc-I (# 3) corresponds to the purified mammalian ST6GalNAc-I (# 3) from submaxillary gland microsomal fractions, it is clear that the avian cloned ST6GalNAc-II enzyme (# 4) is not identical to the mammalian ST6(O)-II (# 5) cloned from rat brain and previously identified in liver microsomal fractions. From an evolutionary point of view, comparison of the amino acid sequences of cloned avian enzymes with their mammalian counterparts reflects the molecular evolution of

sialyltransferases and will provide significant insights in the definition of sialyltransferase sub-families. Furthermore, studies of the sialylated oligosaccharides structures found in inferior vertebrates (i.e. amphibians, fish) have revealed considerable structural variation occurring in the sialylated and polysialylated moieties of membrane glycoproteins. Undoubtedly, these findings will lead to the discovery of new sialyltransferases. Their molecular cloning should be facilitated by the presence of the sialylmotif which has been shown to be conserved in all sialyltransferases cloned to date (Drickamer, 1993; Livingston and Paulson, 1993).

Secondly, as shown in Figure 4, it appears that several distinct sialyltransferases transfer sialic acid in the same linkage onto the same acceptor substrate. This is especially observed for α2,3-sialyltransferases. For instance, three different cloned sialyltransferases (# 6, 7 and 11) are able to transfer sialic acid in α2,3-linkage onto the disaccharide Galβ1-3GlcNAc and three others (# 9, 10 and 11) link sialic acid to the Galβ1-3GalNAc sequence. The very recent work of Chang *et al.* (1995) demonstrates that this latter sub-family of sialyltransferase genes is widely dispersed in the human genome, while ST3GalA.2 (# 10) resides in chromosome 1, ST3(O)-I (# 9) resides in chromosome 8 and ST3(N/O) (# 11) in chromosome 11. Most of the time, a unique transcript arising from the gene gives rise to a unique protein. These genes are differentially expressed in the different cell types and, in that case, the spatio-temporal specific expression of these sialyltransferases is achieved through the level of transcription of one specific gene. In addition, all mammalian and avian α2,3-sialyltransferases appear to transfer sialic acid residues only to galactose residues of *O*-glycans, *N*-glycans and glycolipids. One simplistic explanation would be that α2,3-sialyltransferases recognize other sugar residues, but because of steric hindrance of the *N*-acetyl group, or because of the lack of binding to essential hydrogen bond-forming groups, or even because of the lack of other factors that determine enzyme specificity, α2,3-sialyltransferases transfer sialic acid only onto the C3 of *N*-acetyl sugar derivatives.

In contrast, α2,6-sialyltransferases transfer sialic acid residues onto several different sugar residues such as galactose, *N*-acetylgalactosamine or *N*-acetylglucosamine, but a unique enzyme arising from a single gene synthesizes one sialyl linkage as previously suggested by Roseman (1970). From this single gene, several transcripts are generated from several promoters differentially expressed according to the cell type and giving rise, potentially to several isoforms of the protein. In that case, the specific expression of the enzyme take place through alternative splicing and the use of alternative promoters controlled by tissue-specific transcription factors.

Cell type-specific regulation of the expression of each sialyltransferase gene and their promoter elements remains to be more closely examined in order to understand how the production of cell type-specific carbohydrate structures is regulated. In terms of their evolutionary relationship, sialyltransferase genes have evolved into groups: the α2,3-sialyltransferases, the α2,6-sialyltransferases and the α2,8-sialyltransferases. Expression of these single genes is primarily controlled at the transcriptional level. It depends either on the interaction between *cis*-acting sequences of multiple and distinct promoters with *trans*-acting molecules (tissue-enriched DNA-binding proteins and general transcription factors) resulting in the use of different transcriptional start sites according to the cell type or at different stage of differentiation (α2,6-sialyltransferase) or, alternatively, transcriptional regulation may be achieved through the use of multiple regulatory regions governing the expression of several distinct copies of the same ancestral gene.

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Abbreviations

asialo-G_{M1}, Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-O-Cer; Cer, ceramide; CHO, Chinese hamster ovary cells; CMP, cytidine monophosphate; DBP, D-binding protein; Gal, galactose; Galβ1-O-Cer, galactosylceramide; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine; glucosylceramide, Glcβ1-O-Cer; G_{M1b}, NeuAcα2-3Galβ1-3GalNAcβ1-4Galβ1-4Glcβ1-O-Cer; GRE, glucocortocoid response element; HNF1-α, hepatocyte nuclear factor 1α; Galβ1-4Glcβ1-O-Cer, lactosylceramide; LAP, liver-enriched transcriptional activator protein; Le, Lewis; LT, large tumour; mAb, monoclonal antibody; N-CAM, neural cell adhesion molecule; NeuAc, *N*-acetylneuraminic acid; PCR, polymerase chain reaction; PSA, polysialic acid; PST-1, polysialyltransferase-1; RCA_{120}, *Ricinus communis* agglutinin 120; Ser, serine; ST, sialyltransferase; Thr, threonine; TSH, thyroid-stimulating hormone.

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Very recently, additional sialyltransferases cDNAs have been cloned for various species by means of the PCR approach. In addition, degenerated primers based on two highly conserved regions of the sialylmotif S of mouse ST8Sia-I and ST8Sia-III were used and a cDNA encoding a new α2,8-sialyltransferase (ST8Sia-IV; # 16) was cloned from mouse lung cDNA library (Yoshida *et al.*, 1995b).

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The human sialyltransferase family

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Abstract — The human genome encodes probably more than 20 different sialyltransferases involved in the biosynthesis of sialylated glycoproteins and glycolipids but to date only 15 different human sialyltransferase cDNAs have been cloned and characterized. Each of the sialyltransferase genes is differentially expressed in a tissue-, cell type-, and stage-specific manner to regulate the sialylation pattern of cells. These enzymes differ in their substrate specificity, tissue distribution and various biochemical parameters. However, enzymatic analysis conducted in vitro with recombinant enzyme revealed that one linkage can be synthesized by multiple enzymes. We present here an overview of these human genes and enzymes, the regulation of their occurrence and their involvement in several physiological and pathological processes. © 2001 Société française de biochimie et biologie moléculaire / Éditions scientifiques et médicales Elsevier SAS. All rights reserved.

human genome / sialyltransferase / sialylated glycoproteins / sialylated glycolipids / sialyltransferase cDNA

1. Introduction

In mammals, sialic acids are usually found at the non-reducing terminal position of glycoconjugates sugar chains, α2,3- or α2,6-linked to a β-D-galactopyranosyl (Gal) residue, or α2,6-linked to a β-D-N-acetylgalactosaminyl (GalNAc) residue or a β-D-N-acetylglucosaminyl (GlcNAc) residue. Sialic acids are also found α2,8-linked to sialic acid residues in gangliosides and in polysialic acid (PSA) which is a linear α2,8-homopolymer observed on several glycoproteins (reviewed in [1, 2]). The biosynthesis of sialylated oligosaccharide sequences is catalyzed by a family of enzymes named sialyltransferases [3]. Human sialyltransferases are a functional family of at least 18 different intracellular, Golgi membrane-bound glycosyltransferases (*table I*). In spite of their fundamental role in the synthesis of specific sialylated structures, to date there is still limited information available on their protein structure, mechanism of action and on the cellular mechanisms involved in the regulation of their transcriptional expression. This review summarizes our current knowledge on the structure, enzymatic activity and regulated expression of the cloned human sialyltransferases. We also examine the most recent data on human sialyltransferase gene organization.

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1.1. The sialyltransferase family

To account for all these sialylated structures described to date, the mammalian sialyltransferase family is supposed to consist of more than 20 sialyltransferases, 15 of which have been cloned from human sources (*table I*). However, no cDNA clone has been obtained and characterized yet for the sialyltransferases synthesizing the G_{M4} ganglioside or Neu5Acα2-3Galβ1-4[Neu5Acα2-6]GlcNAc structures. The use of the cloned sialyltransferase cDNAs to produce recombinant proteins has shed light on the substrate specificity of each enzyme in vitro, but it has to be kept in mind that these recombinant enzymes may have a slightly different enzymatic specificity when compared to their purified counterparts.

2. The cloned mammalian sialyltransferases and their substrate specificity

2.1. The α6-sialyltransferases

2.1.1. Sialic acid α2,6-linked to Gal

ST6Gal I mediates the transfer of sialic acid residue with an α2,6-linkage to a terminal Gal residue of type 2 (Galβ1-4GlcNAc) disaccharide found as a free disaccharide or as a terminal *N*-acetyllactosamine unit of an *N*- or *O*-linked oligosaccharides. However, ST6Gal I is unable to use type 1 (Galβ1-3GlcNAc) or type 3 (Galβ1-3GalNAc) structures as acceptor substrate. Weinstein et al.

Table I. Summary of the cloned human sialyltransferase cDNAs and genes.

Sialyltransferases	*Structures formed*	*Chromosomal location*[a]	*Acc. Number*	*cDNA source / gene*	*References*
ST6Gal I (2.4.99.1) SIAT 1	**Neu5Acα2-6**Galβ1-4GlcNAcβ-	3q27q28	X17247 X62822 X54363	placenta gene	5 57 58
ST6GalNAc I (2.4.99.3) Sia7a	$(Neu5Ac\alpha2\text{-}3)_{0\text{-}1}(Gal\beta1\text{-}3)_{0\text{-}1}$GalNAc-Ser / **Neu5Acα2-6**	(17)	Y11339 Y11340 Y11341	Pyloric mucosa	7
ST6GalNAc II Sia7b	$(Neu5Ac\alpha2\text{-}3)_{0\text{-}1}$Galβ1-3GalNAc-Ser / **Neu5Acα2-6**	(17)	AJ251053	MDA-MB 231 epithelial cells	8
ST6GalNAc III (2.4.99.7) Sia7c	Neu5Acα2-3Galβ1-3GalNAc-R / **Neu5Acα2-6**	(1p31.1-31.2)			Human gene not yet cloned
ST6GalNAc IV Sia7d	Neu5Acα2-3Galβ1-3GalNAc-R / **Neu5Acα2-6**	9q34.1	AJ271734 Y17461 Y17460	$HepG_2$ 3 last exons	10 59
ST6GalNAc V $G_{D1\alpha}$ synthase	$G_{D1\alpha}$	(1p31.1)	BE251546 BE083080 AA071204	EST	Human gene not yet cloned
ST6GalNAc VI	$G_{D1\alpha}$, ($G_{T1\alpha}$)	(9)	AB035173	liver	Human gene not yet cloned
ST3Gal I (2.4.99.4) Siat4	**Neu5Acα2-3**Galβ1-3GalNAc-	8q24	L29555 L13972	placenta gene	13 42
ST3Gal II Siat 5	**Neu5Acα2-3**Galβ1-3GalNAc	(16)	U63090 X96667	liver CEM	14 15
ST3Gal III (2.4.99.6) Siat 6	**Neu5Acα2-3**Galβ1-3/4GlcNAcβ-	1(p34p33)	L23768	placenta	16
ST3Gal IV (2.4.99.4) Siat-4c, STZ	**Neu5Acα2-3**Galβ1-4GlcNAc[b] **Neu5Acα2-3**Galβ1-3GalNAc	11(q23-q24)	L23767 X74570 L29553	placenta B. lymphoma gene	17 18 43
ST3Gal V (2.4.99.9) G_{M3} synthase, Siat 9	**Neu5Acα2-3**Galß1-4Glc-Cer	(2)	AB018356	HL-60	19
ST3Gal VI	**Neu5Acα2-3**Galβ1-4GlcNAcβ-	(3)	AB022918	SK-MEL-37	22
ST8Sia I (2.4.99.8) SAT-II, G_{D3}synthase, SIAT 8a	**Neu5Acα2-8**Neu5Acα2-3Galβ1-4Glc-Cer	12p12	D26360 X77922 L32867 L43494	melanoma melanoma melanoma gene	24 25 23 26
ST8Sia II STX, SIAT 8b	**Neu5Acα2-8**Neu5Acα2-3Galβ1-4GlcNAc	15q26	L29556 U33551 U82762	placenta small cell lung carcinoma gene	27 28 29
ST8Sia III SIAT 8c	**Neu5Acα2-8**Neu5Acα2-3Galβ1-4GlcNAc	(18)	AF004668	brain	30
ST8Sia IV PST-1, SIAT 8d	**Neu5Acα2-8**$(Neu5Ac\alpha2\text{-}8)_n$Neu5Acα2-3Galβ1-R	5q21	L41680	fetal brain gene	32 29
ST8Sia V SAT-V	G_{D1c}, G_{T1a}, G_{Q1b}, G_{T3}.	18	U91641	brain	33

[a] Chromosomal locations indicated in parentheses have been determined by sequence comparison in genomic databases.
[b] Refers to the preferred acceptor.

have isolated the first cDNA clone of a sialyltransferase from the rat species in 1987 [4] and Grundmann et al. the first human cDNA in 1990 [5].

2.1.2. Sialic acid α2,6-linked to GalNAc

So far, the cloning and characterization of three members of the human GalNAc α2,6-sialyltransferase family (hST6GalNAc I, II and IV) have been reported. However, six different GalNAc α2,6-sialyltransferases are known to exist in mouse [6].

As far as seen with the expressed mouse and human recombinant enzymes in in vitro assays, three of them (ST6GalNAc I, II and IV) catalyze the formation of α2,6-linkages onto GalNAc residues *O*-glycosidically linked to Ser/Thr (*figure 1*), and three others (ST6GalNAc III, V and VI) catalyze the addition of sialic acid residues onto gangliosides (*figure 2*). Human ST6GalNAc I exhibits the broadest specificity for the following structures : GalNAcα-*O*-Ser/Thr, Galβ1-3GalNAcα-*O*-Ser/Thr and Neu5Acα2-3Galβ1-3GalNAcα-*O*-Ser/Thr [7]. Human

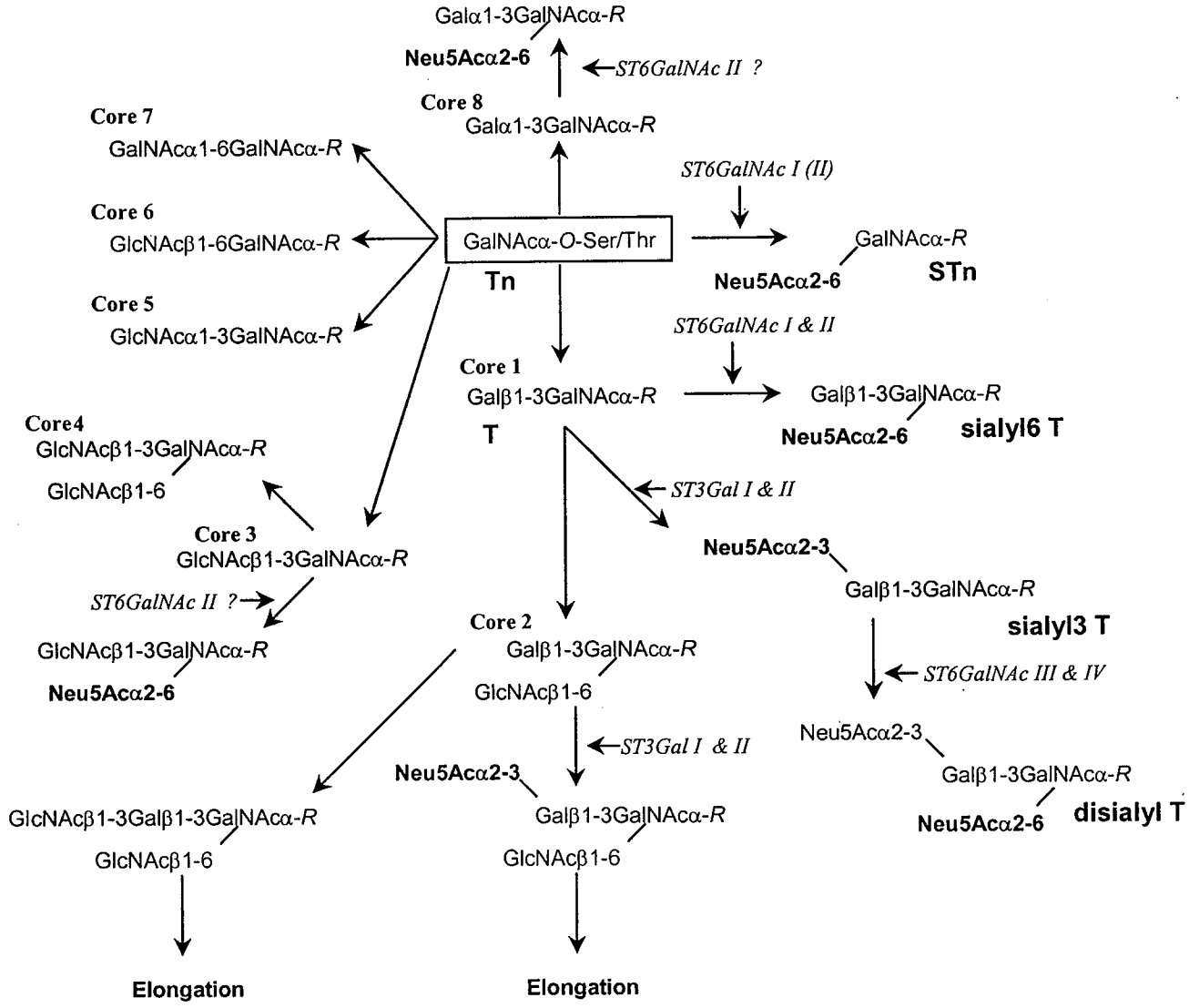

Figure 1. Sialylation reactions in the initial steps of the *O*-glycans biosynthesis. The name of the compound, if any, is indicated underneath the glycan structure. The sialic acid residue transferred is indicated in bold characters. The enzymes are indicated in italic and the question mark indicates that the enzyme is not characterized.

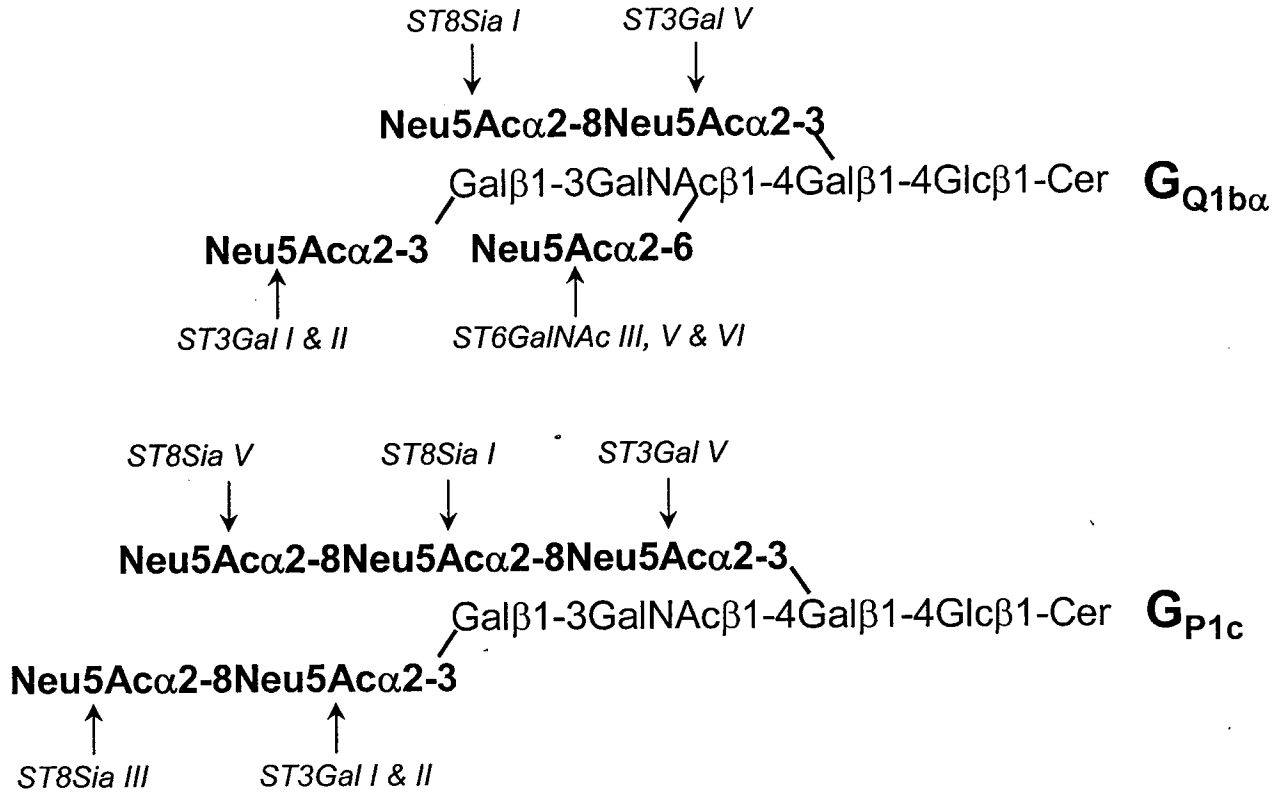

Figure 2. Gangliosides biosynthesis. Schematic representation of the sialyltransferases (in italic) involved in the biosynthesis of gangliosides. The sialic acid residues transferred are indicated in bold characters.

ST6GalNAc II exhibits a narrower substrate specificity using Galβ1-3GalNAcα-*O*-Ser/Thr and Neu5Acα2-3Galβ1-3GalNAcα-*O*-Ser/Thr as substrates [8]. The human ST6GalNAc II gene is expressed at low levels in heart, skeletal muscle, kidney and liver. The mouse ST6GalNAc III and human ST6GalNAc IV exhibit a most restricted substrate specificity, only utilizing the Neu5Acα2-3Galβ1-3GalNAc trisaccharide sequence found on either *O*-glycosylproteins or ganglioside G_{M1b}, suggesting that they do not discriminate between α- and β-linked GalNAc [9, 10]. No cDNA clone for human ST6GalNAc III has been obtained or characterised yet, and no EST sequence is found in databases (*table I*).

Mouse ST6GalNAc V, expressed only in the brain, seems to be specific for G_{M1b} [11, 12]. Mouse ST6GalNAc VI [6] is expressed in a wide range of mouse tissues such as colon, liver heart, spleen and brain. This enzyme appears to be specific for glycolipid acceptors and can synthesise all α-series gangliosides defined so far (*figure 2*). For these last two enzymes, no human clones have been published and characterized yet but ESTs are available in public databases (*table I*).

2.2. The α3-sialyltransferases

Six different α2,3-sialyltransferase cDNAs have been cloned from human cells or tissues and enzymatically characterized. However, the reported substrate specificity of these various recombinant enzymes remains somewhat unclear.

Two Galβ1-3GalNAc α2,3-sialyltransferases (ST3Gal I and ST3Gal II) mediate the transfer of sialic acid residues to a Gal residue of terminal Galβ1-3GalNAc oligosaccharide found on glycolipids (asialo-G_{M1} and G_{M1a}) or glycoproteins (*figures 2, 3*) [13–15].

Human Galβ1-3(4)GlcNAc α2,3-sialyltransferase (hST3Gal III) cloned by Kitagawa and Paulson [16] preferentially acts on type 1 chain (Galβ1-3GlcNAc) and is therefore the candidate for the synthesis of sialyl-Lewis^a epitope, in vivo. ST3Gal III can also catalyze the sialylation of type 2 chain (Galβ1-4GlcNAc) but with lower catalytic efficiency. ST3Gal III gene has been shown to be highly expressed in skeletal muscle but not in placenta suggesting the existence of a second enzyme with similar specificity.

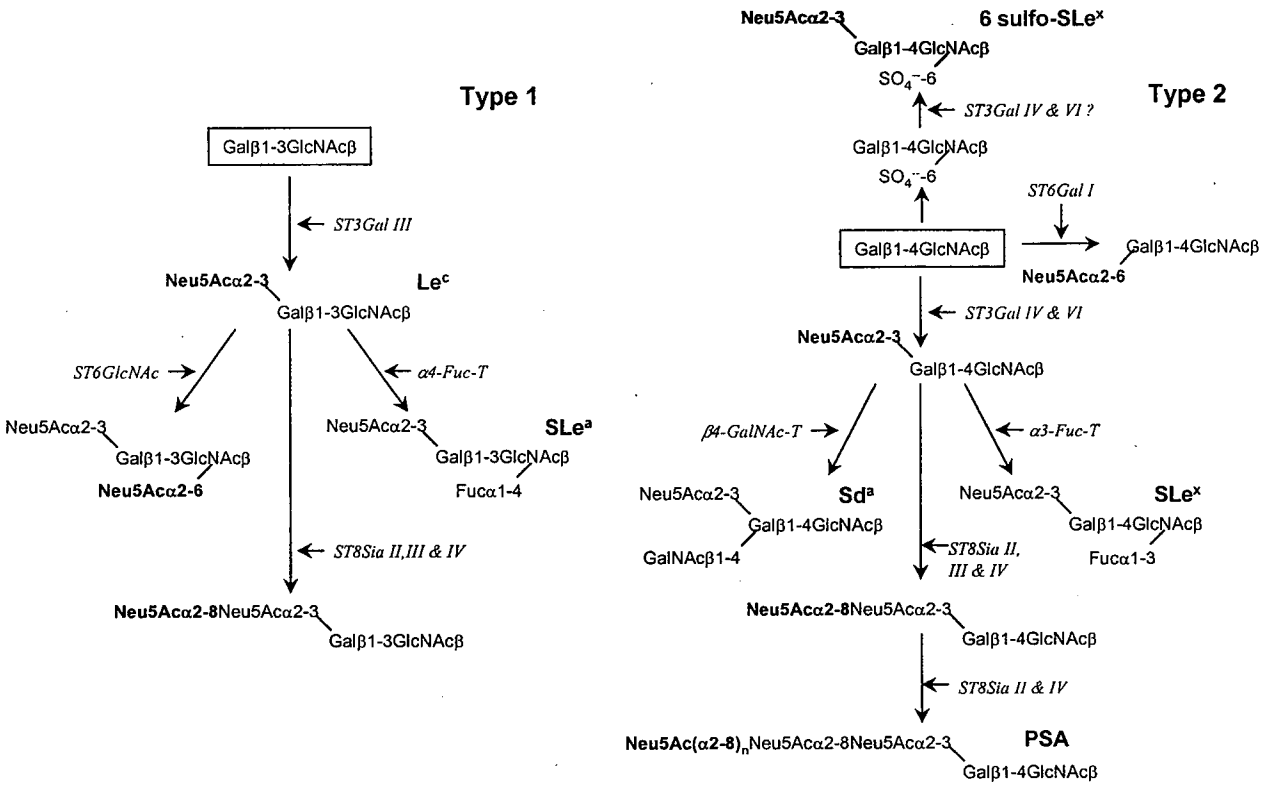

Figure 3. Terminal sialylation reactions on type 1 and type 2 glycan chains. The name of the antigen is indicated next to the glycan structure. The sialic acid residue transferred is indicated in bold characters. The enzymes are indicated in italic and the question mark indicates that the enzyme is not characterized.

A human Galβ1-4GlcNAc α2,3-sialyltransferase cDNA (hST3Gal IV) has been cloned independently from human placenta [17] and from the human Burkitt lymphoma cell line Namalwa [18]. Analyses using recombinant soluble ST3Gal IV have shown that this enzyme used either glycolipids or glycoproteins containing Galβ1-4GlcNAc or Galβ1-3GalNAc sequences. Human ST3Gal IV transcripts are abundantly expressed in placenta [17].

Recently, the cloning of the human G_{M3} synthase cDNA (hST3Gal V) has been reported by Ishii et al. [19]. The recombinant human enzyme was shown to use only lactosylceramide (Galβ1-4Glcβ-Cer) as an acceptor substrate leading to the synthesis of G_{M3} (Neu5Acα2-3Galβ1-4Glcβ-Cer) whereas the purified rat liver enzyme exhibited a broader specificity utilizing both galactosylceramide (Galβ-Cer) and asialoganglioside G_{A2} (GalNAcβ1-4Galβ1-4Glcβ-Cer) as acceptor substrate as well as lactosylceramide (Galβ1-4Glcβ-Cer) [20, 21]. Despite the ubiquitous distribution of G_{M3} in human tissues, the ST3Gal V gene was found to be expressed in a tissue-specific manner, with predominant expression in brain, skeletal muscle and testis. Very low levels of ST3Gal V mRNA were found in liver.

Human ST3Gal VI [22] utilizes almost exclusively Galβ1-4GlcNAc on glycoproteins and glycolipids (*figure 3*). Among glycolipid acceptors, ST3Gal VI preferred polylactosamine type 2 chains thus generating glycolipids containing Neu5Acα2-3Galβ1-4GlcNAc structures including sialyl-paragloboside, a precursor of the sialyl-Leˣ on ceramide. Predominant expression of the ST3Gal VI gene was found in placenta, liver, heart and skeletal muscle.

2.3. The α8-sialyltransferases

The human sialylα2-3Galβ1-4Glcβ1-*O*-Cer α2,8-sialyltransferase (hST8Sia I) catalyzes the synthesis of G_{D3} (Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ1-*O*-Cer) and the corresponding human cDNA has been cloned by three different groups [23–25]. Later on, it was found by Nakayama et al. [26], that G_{D3} and G_{T3} (Neu5Acα2-8Neu5Acα2-8Neu5Acα2-3Galβ1-4Glcβ1-*O*-Cer) structures were synthesized by this single enzyme. No sialyltransferase activity was found towards glycoproteins. The G_{D3}/G_{T3} synthase gene is expressed at very low level in fetal brain and fetal lung.

The sialylα2-3Galβ1-4GlcNAc-R α2,8-sialyltransferase (ST8Sia II) has been described at first as the initiating enzyme that transfers the first sialic acid residue to the 8-hydroxyl of Neu5Ac residue α2,3-linked to the terminal position of *N*-linked glycans [27] mainly found attached to the neural cell adhesion molecule N-CAM. A human cDNA clone corresponding to this enzyme has been obtained by Scheidegger et al. [28], but no activity was observed towards glycolipids including G_{M3} and sialylparagloboside. The human ST8Sia II gene is expressed in fetal brain and kidney but also in adult heart, thymus and brain [29].

The cDNA encoding another human sialylα2-3Galβ1-4GlcNAc-R α2,8-sialyltransferase (hST8Sia III) was isolated from a brain cDNA library [30]. The recombinant soluble human ST8Sia III protein expressed in COS-7 cells showed a high catalytic activity of transfer of sialic acid residue to intact fetuin. However, the murine recombinant enzyme synthesizes oligomeric α2,8-sialic acid linkage on *N*-glycans and to a lesser extent on glycolipids but does not form polysialic acid [31]. Northern analyses have shown that corresponding transcripts were expressed in fetal and adult brain and liver.

A fourth human α2,8-sialyltransferase (hST8Sia IV) has been cloned that shows polysialic acid synthase activity in vitro towards N-CAM [32]. Human ST8Sia IV gene has been found to be expressed in fetal brain, kidney and lung and in adult spleen, thymus, heart, small intestine and leukocyte.

The molecular cloning of a fifth type of human α2,8-sialyltransferase (ST8Sia V) has been reported by Kim et al. [33]. As previously shown for the mouse ST8Sia V [34], the recombinant enzyme exhibited activity towards gangliosides, G_{M1b}, G_{D1a}, G_{T1b}, and G_{D3}. Human ST8Sia V is expressed in both human fetal and adult brain, but also in adult heart and skeletal muscle.

3. Structure of sialyltransferases

Analysis of the deduced protein sequence indicated that sialyltransferases are type II transmembrane glycoproteins with a short NH_2-terminal cytoplasmic tail which is not essential for catalytic activity, and a 16–20 amino acid signal anchor domain that participates to the retention signal for these Golgi luminal enzymes. The stem region, highly variable in length (from 20 amino acids to 200 amino acids) is followed by a large COOH-terminal catalytic domain that resides in the lumen [35]. Despite sharing this topological similarity, the amino acid sequences of the human cloned sialyltransferases show very little sequence identity with the exception of the three consensus sequences called sialylmotifs L, S, and VS (*figure 4*). The functional significance of the sialylmotifs L and S has been assessed by site-directed mutagenesis using ST6Gal I as a model. Data obtained provided evidence that the L-sialylmotif participates in the binding of the sugar donor CMP-Neu5Ac [36] whereas the S-sialylmotif participates in the binding of both the donor and the acceptor substrates [37]. Each of the two sialylmotifs contains one cysteine residue invariantly present in all cloned sialyltransferases (*figure 4*). Preliminary data have shown that these cysteine residues are important for the enzyme activity and are involved in disulfide linkage formation [37]. We have also identified two conserved amino acid residues, a Glu (E) residue separated by four amino acid residues from an His (H) present at the C-terminus in all sialyltransferases sequences. This motif was named VS and may be involved in the catalytic process, although no experimental data have yet confirmed this hypothesis [38]. Finally, protein sequence analysis among the members of each subfamily (α2,3-, α2,6-, α2,8-sialyltransferases) revealed that each of these shares a common sequence near the 3'-end of the sialylmotif S (*figure 4*). For example, members of the ST8Sia subfamily share a conserved amino acid sequence (I/L)(F/Y)GFWPF at the 3' end of the S-sialylmotif. Members of the α3-sialyltransferase subfamily share the sequence (Y/A)GF(K/G)(Y/A) and the α6-sialyltransferase subfamily, the sequence (A/V)YG(F/M) [37, 39]. In addition, all sialyltransferases share another motif (Y/H)Y(W/F/Y)(D/E/G/Q) between the sialylmotif S and VS [40]. The functional role of these motifs is still obscure. Based on computer modeling, it has been speculated that they could be involved in interaction with the cytosine moiety of CMP-Neu5Ac [40]. Finally, as discussed in our recent review [2], analysis of the canonical sequences for post-translational modifications in primary amino acid sequences of the sialyltransferases revealed several consensus sites for phosphorylation and *N*-glycosylation. These post-translational modifications may influence the catalytic activity and intracellular traffic of sialyltransferases.

4. Sialyltransferase gene organization

Recently, we have performed an extensive search of GeneBank/EMBL human genome sequence database (HTG) with the TBLASTN algorithm which enabled us to assemble several sequences of putative processed and unprocessed sialyltransferases genes. However, whether this represents functional genes or the remnants of once functional genes remains to be demonstrated. This analysis allowed us also to report for the first time on the chromosomal location and gene organization of all human sialyltransferases (*figure 5*). In some cases, the human cDNA has not been published (hST6GalNAc III, V and VI) but their mouse homologues were used as probe to screen the databanks.

The genomic organization and chromosomal assignment of five human sialyltransferase genes have been

Enzyme	*Sialylmotif L*
hST6Gal I	WGRCAVVSSAGSLKSSQLGREIDDHDAVLRFNGAPTANFQQ-DVGTKTT
hST6GalNAc I	CITCAVVGNGGILNNSHMGQEIDSHDYVFRLSGALIKGYEQ-DVGTRTS
hST6GalNAc II	CIRCAVVGNGGILNGSRQGPNIDAHDYVFRLNGAVIKGFER-DVGTKTS
hST6GalNAc III	CDLCAIVSNSGQMVGQKVGNEIDRSSCIVRMN-APTRGYEE-DVGRMNT
hST6GalNAc IV	CRSCAVVSSSGQMLGSGLGAEIDSAECVFRMNQAPTVGFEA-DVGQRTT
hST6GalNAc V	KCTCALVTSSGHLLHSRQGSQIDQTECVIRMNDAPTRGYGR-DVGNRTS
hST6GalNAc VI	CHQCVIVSSSSHLLGTKLGPEIERAECTIRMNDAPTTGYSA-DVGNKTT
hST3Gal I	CRRCAVVGNSGNLRESSYGPEIDSHDFVLRMNKAPTAGFEA-DVGTKTT
hST3Gal II	CRRCAVVGNSGNLRGSGYGQDVDGHNFIMRMNQAPTVGFEQ-DVGSRTT
hST3Gal III	CRRCIIVGNGGVLANKSLGSRIDDYDIVVRLNSAPVKGFEK-DVGSKTT
hST3Gal IV	CRRCVVVGNGHRLRNSSLGDAINKYDVVIRLNNAPVAGYEG-DVGSKTT
hST3Gal V	CRRCVVIGSGGILHGLELGHTLNQFDVVIRLNSAPVEGYSE-HVGNKTT
hST3Gal VI	CKKCVVVGNGGVLKNKTLGEKIDSYDVIIRMNNGPVLGHEE-EVGRRTT
hST8Sia I	LKKCAVVGNGGILKKSGCGRQIDEANFVMRCNLPPLSSEYTKDVGSKSQ
hST8Sia II	FQTCAIVGNSGVLLNSGYGQEIDAHSFVIRCNLAPVQEYAR-DVGLKTD
hST8Sia III	YNICAVVGNSGILTFIQCGREIDKSDFVFRCNFAPSEAFQR-DVGRKTN
hST8Sia IV	FKTCAVVGNSGILLDSECGKEIDSHNFVIRCNLAPVVEFAA-DVGTKSD
hST8Sia V	FKKCAVVGNGGILKNSRCGREINSADFVFRCNLPPISEKYTMDVGVKTD
	* * * **

Enzyme	*Sialylmotif S*	*Sialylmotif VS*
hST6Gal I	PSSGMLGIIIMMTLCDQVDIYEFLPSKRKTDVC------YYYQKFFDSACTMGAY---------HPLLYEK	
hST6GalNAc I	PTTGALLLLTALQLCDQVSAYGFITEGHERFSD------HYYDTSWKR----------LIFYINHDFKLER	
hST6GalNAc II	PSTGALMLLTALHTCDQVSAYGFITSNYWKFSD------HYFERKMKP----------LIFYANHDLSLEA	
hST6GalNAc III	LSSGWFTFILAMDACYGIHVYGMMNFFSRTEGYRK-VPYHYYEQGR-DECDEYFLHEHAP-G-GHRFITEK	
hST6GalNAc IV	• **LSTGWFTMILALELCEEIVAYGMVSDSYCREKSHPSVPYHYFEKGRLDECQMYLAHEQAR--SAHRFITEK**	
hST6GalNAc V	• **LSTGWFTMTIALELCDRINVYGMVPPDFCRDPNHPSVPYHYYEPFGPDECTMYLSHERGRKGSHHRFITEK**	
hST6GalNAc VI	• **LSTGWFTMVIAVELCDHVHVYGMVPPNYCSQRPVQRMPYHYYEPKGPDECXTYIQNEHSRKGNHHRFITEK**	
hST3Gal I	PSTGILSVIFSMHVCDEVDLYGFGADSKGNWH-------HYWENNPSAGAFRKTGV--------HDADFES	
hST3Gal II	PSTGMLVLFFALHVCDEVNVYGFGADSRGNWH-------HYWENNRYAGEFRKTGV--------HDADFEA	
hST3Gal III	PTLGSVAVTMALHGCDEVAVAGFGYDMSTPNAPL-----HYYETVRMAAIKESWT---------HNIQREK	
hST3Gal IV	PTTGLLAITLALHLCDLVHIAGFGYPDAYNKKQTI----HYYEQITLKSMAGSG----------HNVSQEA	
hST3Gal V	PTIGVIAVVLATHLCDEVSLAGFGYDLNQPRTPL-----HYFDSQCMAAMNFQTM---------HNVTTET	
hST3Gal VI	PTTGIIAITLAFYICHEVHLAGFKYNFSDLKSPL-----HYYGNATMSLMNKNAY---------HNVTAEQ	
hST8Sia I	LSTGLFLVSAALGLCEEVAIYGFWPFSVNMHEQPIS--HHYYDNVLPFSGF-------------HAMPEEF	
hST8Sia II	PTTGLLMYTLATRFCKQIYLYGFWPFPLDQNQNPVK--YHYYDSLKYGYTSQASP---------HTMPLEF	
hST8Sia III	LSTGILMYTLASAICEEIHLYGFWPFGFDPNTREDLP-YHYYDKKGTKFTTKWQES---------HQLPAEF	
hST8Sia IV	PSTGLLMYTLATRFCDEIHLYGFWPFPKDLNGKAVK--YHYYDDLKYRYFSNASP---------HRMPLEF	
hST8Sia V	ISTGLILVTAALELCEEVHLFGFWAFPMNPSGLYIT--HHYYDNVKPRPGF-------------HAMPSEI	
	* * * * *	

Figure 4. Sialylmotifs L, S and VS of human sialyltransferases. The human sialyltransferase protein sequences were aligned. The stars indicate amino acids strictly conserved.

described to date: hST6Gal I [41], hST6GalNAc IV [10], hST3Gal I, II and IV [42]. Sialyltransferases are dispersed among multiple exons (*figure 5*) and are widely dispersed in the human genome with the notable exception of the six described ST6GalNAc genes that are found by pairs on three chromosomes (1, 9 and 17). This observation suggests that these latter genes originated by tandem duplication from a single ancestral gene. Comparison of exon/intron boundaries and exons sizes shows that sialyltransferases have a similar genomic structure and are related two by two, with the notable exception of ST6Gal I and ST8Sia III. Closely related sialyltransferases such as

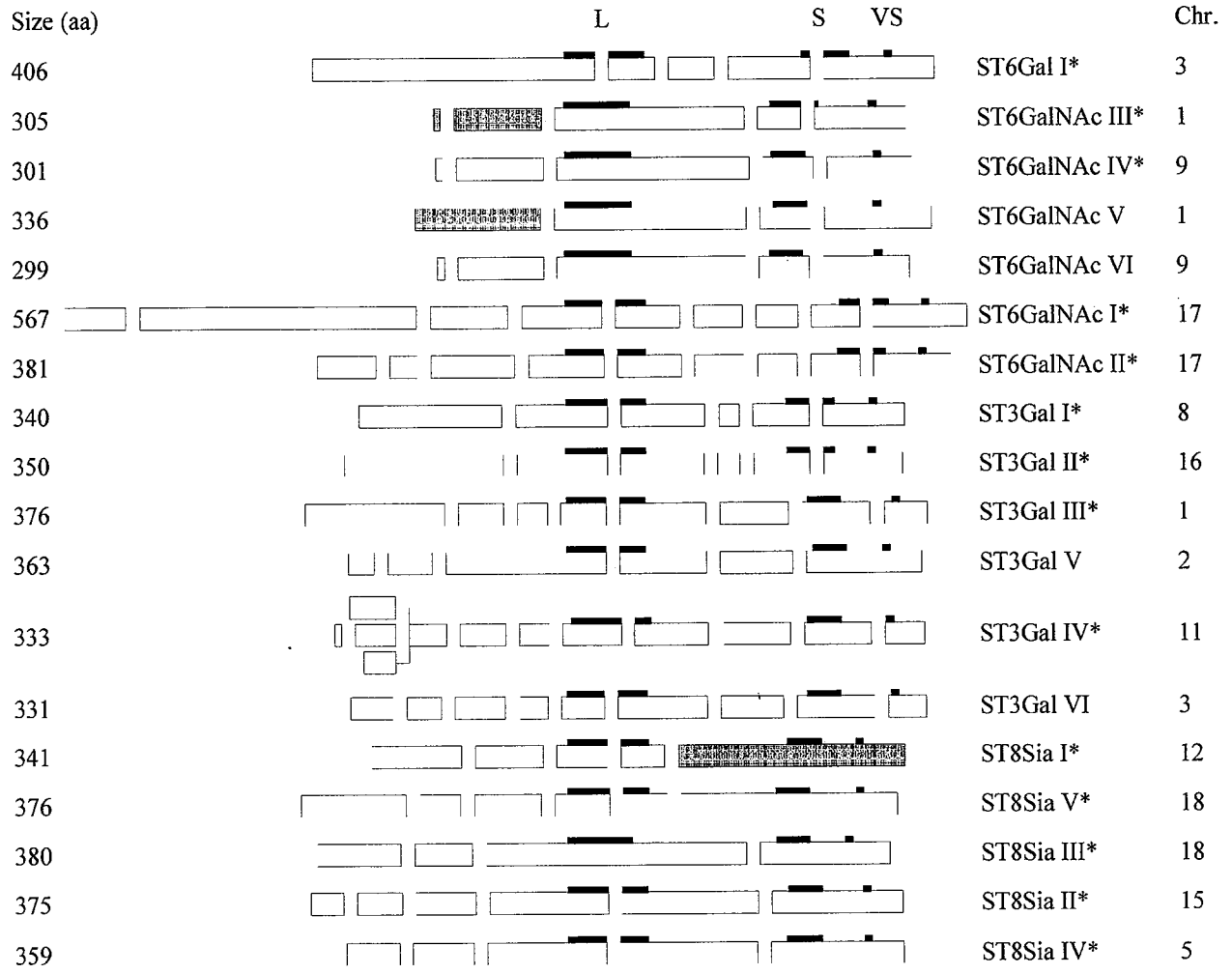

Figure 5. Exon/intron organization of human sialyltransferase genes. Open boxes represent protein coding sequence exons and the black lines the sialylmotifs. Gray boxes indicate exons which have not be characterized in human genome. Stars indicate the enzymes for which the mouse genomic organization has been reported. L, S, VS, large, small, and very small sialyl motifs, respectively; Chr, chromosome location.

ST3Gal I and ST3Gal II are not localized on the same chromosome suggesting that they have arisen from duplication and subsequent translocation events.

5. Regulation of the sialyltransferase gene expression

The relative activity of sialyltransferases influences the expression of sialylated compound at the cell surface and contributes to the definition of the glycosylation pattern of normal and tumor cells. Sialyltransferase spatio-temporal expression appears to be regulated mainly at the level of transcription which has been evidenced through Northern blot and in situ hybridization experiments by a strong positive correlation between mRNA expression levels and enzyme activity levels (reviewed previously in [2]). Towards the elucidation of the molecular basis of human sialyltransferase gene expression, the genomic regions containing the promoter were isolated and functionally characterized for only two human sialyltransferases genes, hST6Gal I and hST3Gal IV. The sequence analysis of the 5'-flanking region of the two genes revealed the heterogeneous transcription start sites and the absence of the canonical TATA and CCAAT boxes coupled with the presence of several GC boxes. These structural features are typical of the housekeeping genes, which are ex

pressed in essentially all tissues. In vivo, these multiple mRNA forms, differing only in their 5'-untranslated region are expressed in a tissue-specific manner. This mRNA heterogeneity has been attributed, at least in part, to transcription from a number of physically distinct promoter regions that govern and regulate their expression in specific cell types.

The ST3Gal IV gene transcription results in five distinct mRNAs (A1, A2, B1, B2, B3) which are generated by alternative splicing and alternative promoter usage [43]. Taniguchi et al. have recently described an epithelial-cell-specific transcriptional regulation of the human hST3Gal IV gene expression and delineated a functional DNA portion upstream of the gene (promoter B3) containing two sequences similar to AP2 sites [44]. The same authors have reported the down-regulation of hST3Gal IV gene expression during human keratinocyte cell differentiation [45]. Five different transcripts of hST3Gal IV have been identified in leukaemic cell lines and leucocytes [46] and their expression has been shown to be controlled at least in part by the AP1 transcription factor. In HL-60 cell line, the expression levels of two of these transcripts changed during differentiation, leading to the down-regulation of hST3Gal IV expression [47].

Multiple mRNA isoforms are generated from the gene encoding ST6Gal I. These isoforms, transcriptionally initiated from a number of physically distinct promoter regions, differ only in their 5'-most untranslated region and share an identical ST6Gal I coding region. A short mRNA form (form I) has been isolated from liver and initially characterized from $HepG_2$ hepatoma cells [48, 49]. A large transcript (form 3) containing two 5'-untranslated exons (exons Y+Z) has been isolated from several cell types. Taniguchi et al. have studied the transcriptional regulation of ST6Gal I gene during HL-60 differentiation and suggested that SP1and Oct-1 sites involved in myeloid cell specific gene regulation, may play a critical role [50]. A distinct transcript (form 2) containing one 5'-untranslated exon (exon X) but not exons Y+Z has been isolated from human B cells lymphoblastoma cell line. In mature B-lymphocytes, cell type-specific expression correlates with presence of this divergent form 2 mRNA [51]. During neoplastic transformation of colonic cells, ST6Gal I expression is being modulated through a differential promoter usage and the differential expression of the hepatic transcript [52, 53].

Investigations concerning the physiological roles of some of the key members of the sialyltransferase genes family via their gene disruption in mice as well as the mechanisms by which their dysfunction initiate disease are quite recent since these genes have only recently become available for study [54]. As a first example, inactivation of the ST6Gal I gene in mice has shown that its function on *N*-glycans is essential for B lymphocyte immune function with impaired B cell proliferation and IgM production, but without any other apparent abnormalities in physiology, morphology or behavior [55]. As another example, ST3Gal I controls CD8+ T lymphocyte homeostasis by modulating *O*-glycan biosynthesis. Null mutation results in a deficiency of cytotoxic CD8+ T lymphocytes by apoptosis [56].

6. Conclusion

During the past decade, 18 different sialyltransferase cDNAs have been cloned from different mammalian species, allowing the production of large number of recombinant enzymes. These were expressed as either full-length membrane-bound or soluble enzymes, in various host cells including mammalian cells, insect cells, yeast and *E. coli.* This allowed determining the in vitro specificity of each individual sialyltransferase, but the physiological meaning of these data remains to be demonstrated in vivo. Deletion of sialyltransferase genes from the mouse genome provide another powerful tool to gain access to the biological function of sialylglycoconjugates. Expression of each sialyltransferase gene is strictly regulated in a cell type and development specific manner. Analysis of the promoter regions of the sialyltransferase genes will be a main goal for the next decade towards the understanding of the regulatory mechanism of their expression in physiological and pathological conditions.

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REVIEW

GLYCOSIDASES

(FUCOSIDASES, GALACTOSIDASES, GLUCOSIDASES, HEXOSAMINIDASES AND GLUCURONIDASE FROM SOME MOLLUSCS AND VERTEBRATES, AND NEURAMINIDASE FROM VIRUS)

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1. INTRODUCTION

Glycosidases or carbohydrases are enzymes, included in class 3 of the Enzyme classification (IUB, 1979), that catalyze the hydrolysis of glycoconjugates (glycoproteins, glycolipids and glycosaminoglycans) as well as synthetic substrates which contain glycosidic linkages.

Some glycosidases are endoglycosidases i.e. they split internal linkages and liberate oligosaccharides, however, there is a higher number of glycosidases, named exoglycosidases, that act at the end of the carbohydrate chain and release monosaccharides.

The empiric use of some glycosidases is very old. Man has taken advantage of the transformation of some materials such as starch to produce glucose after hydrolysis catalyzed by glycosidases. Kirchoff, in 1814, studied the degradation of starch in sugar by the malt extract which he named "diastase". Payen & Persoz purified the diastase from malt and also discovered this enzyme in saliva. Robiquet, in 1838, studied the hydrolysis of the glucoside amygdalin by the "albuminoïde" substance ("emulsin") contained in the bitter almonds. At the end of the Nineteenth century, the studies of E. Fischer, and later, in the twenties of the present century, those carried out by von Euler, Kuhn & Willstätter were mainly concerned with a preliminary approach to the glycosidases (for a summary on the history of enzymes, see Robert & Polonovski, 1964; Villar-Palasi *et al.*, 1977).

Between approximately 1925 and 1950 the research on glycosidases decreased, however, after 1950 it has been intensely developed, mainly in fields such as microorganisms, molluscs and mammals.

Courtois and collaborators, who have studied several glycosidases in the fifties and later pursued the inheritance of the French scientists such as Herissey & Fleury, have reviewed, twenty years ago, the studies on glycosidases (Courtois & Perlés, 1964).

Some of the *reasons that may explain the emergence of a new knowledge on glycosidases* after the decade of 1950, are the following:

(a) the large distribution of these enzymes in Nature (actually considered as ubiquitous), which indicate their relevant role;

(b) the demonstration of the occurrence of glycosidases mainly in lysosomes, but also in other subcellular fractions;

(c) the fact that an exoglycosidase from *Escherichia coli*, the β-galactosidase, has been employed in molecular biology, for informationists studies, as well the lysozyme has been investigated in molecular biology for the structuralist approach;

(d) the use of several glycosidases, either partially or completely purified, as tools for the determination of glycoconjugates structures;

(e) the characterization of inherited metabolic disorders in which there is deficiency either of one glycosidase or one glycosidase isoenzyme;

(f) finally, the recent knowledge on the role of some monosaccharides in the half-life of the plasma glycoproteins, regulated by the liberation of these carbohydrates by some glycosidases.

It explains that the number of papers on glycosidases has intensely increased after 1953.

The following *characteristics* are typical of many glycosidases:

(a) generally, they have acidic pH optima, thus, they are named "acid" glycosidases;

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Abbreviations: Neu5Ac, *N*-acetylneuraminic acid; Neu5Gl, *N*-glycolylneuraminic acid; Neu4,5Ac$_2$, 4-*O*-acetyl-*N*-acetylneuraminic acid; Neu5AcylxAc, *O*-acetylated *N*-acetyl or *N*-glycolylneuraminic acid where the position of the *O*-acetyl group(s) is unknown; GM_1, II^{3}NeuAc-$GgOse_4Cer$; GM_3, II^{3}NeuAc-LacCer; GD_3, II^3(NeuAc)$_2$-LaCer; GD_{1a}, IV^{3}NeuAc, II^{3}NeuAc-$GgOse_4Cer$; GD_{1b}, II^3(NeuAc)$_2$-$GgOse_4Cer$; GT_{1b}, IV^{3}NeuAc, II^3(NeuAc)$_2$-$GgOse_4Cer$.

(b) they occur mainly in lysosomes (about 50 hydrolases have been found in this organelle). However there are some that occur also in non-lysosomal locations: β-D-glucuronidase in both lysosomes and endoplasmic reticulum; α-D-mannosidase in lysosomes, Golgi membranes and cytosol; neuraminidase in lysosomes, Golgi membranes, cytosol and plasma membrane of rat liver (Strawser & Touster, 1980);

(c) it seems that other lysosomal enzymes are glycoproteins, except cathepsin B_1 and lysozyme, and many of them, sialoglycoproteins (Strawser & Touster, 1980);

(d) many glycosidases show multiplicity of forms, from genetic or nongenetic origin and are capable of hydrolyzing the same substrate. Since the amino acid sequences of these enzymes are generally not determined yet, the multiplicity of forms is only partially understood;

(e) in contrast to the fact of multiplicity of forms, some glycosidases (such as β-galactosidase from various materials) have the property of hydrolyzing several substrates which differ by the D-glycosidically bound sugar (see later).

The purification of glycosidases is in many cases a tedious job, like for other enzymes. Actually, affinity chromatography procedures are employed for purification of glycosidases from several materials. Isoelectric focusing contributes to the characterization and purification of these enzymes. However, some modifications and loss of activity may occur.

Taking into consideration that only a relatively reduced number of glycosidases have been purified, the absolute acceptation of some data on glycosidases should require a confirmation.

2. REVIEWS ON GLYCOSIDASES

The following comments mainly concern glycosidases from animal sources. Those on microbial glycosidases will not be included, except for neuraminidase.

β-Galactosidase has been reviewed by several authors after 1960 (see Wallenfels & Malhotra, 1960, 1961; Wallenfels & Weil, 1972) and recently by Richmond *et al.* (1981). Its purification by affinity techniques has also been described (Steers & Cuatrecasas, 1974).

α-Galactosidase has been reviewed and we dispose of the publication of Wallenfels & Malhotra (1961) and that of Dey & Pridham (1972). Its purification by affinity chromatography has also been reported (Harpaz & Flowers, 1974).

Studies on human α-galactosidases and other α-glycosidases has been the subject of a Doctoral Thesis (Schram, 1978).

Furthermore, data concerning *both galactosidases* and other glycosidases has been studied by authors such as Egami (1974), Buddecke & Werries (1974), Hatcher & Jeanloz (1974), Li & Li (1974) and Yamashina *et al.* (1974), mainly in which concerns the use of these enzymes as tools for elucidation of the structure of complex carbohydrate chains.

Reviews on *hexosaminidases* (Lowden *et al.*, 1981), molluse hexosaminidases (Calvo, 1977), human *α-L-fucosidases* and fucosidosis (Alhadeff & O'Brien, 1977; Alhadeff, 1981), and the role of α-L-fucosidases in the catabolism of blood group substances (Wiederschain & Rosenfeld, 1974) have appeared.

A general review on *glycosidases* (mainly concerning their action on natural substrates) is that of Flowers & Sharon (1979).

The purification and properties of *several mammalian glycosidases* have been described by Touster (1978), Distler & Jourdian (1978), Furbish *et al.* (1978), Kusiak *et al.* (1978), Geiger & Arnon (1978), Tarentino & Maley (1978) and Lisman (1974). Furthermore, topics such as the cellular localization (mainly in lysosomes) of many glycosidases have been reviewed by Barrett (1972), Brunngraber (1979) and Strawser & Touser (1980). Their role in the glycoproteins catabolism (Patel & Tappel, 1971), glycolipid catabolism (Dawson, 1978), as well as in glycoproteinoses (Strecker & Montreuil, 1979; Montreuil, 1981) has been intensely studied. Besides, several papers on glycolipids hydrolases have also appeared (see Dean *et al.*, 1978; Schram *et al.*, 1978; Li *et al.*, 1978; Yeung *et al.*, 1978; Dubois & Baumann, 1978; Jatzkewitz, 1978; Leibovitz *et al.*, 1978; Radin, 1978), as well as on sphingolipidoses (see Glew & Peters, 1977) and mucopolysaccharidoses (Kresse *et al.*, 1982).

On the other hand, several reviews on *neuraminidase* or *sialidase* from various sources (viruses, bacteria, mammals) have appeared after 1966 (see a list of a dozen references in Cabezas *et al.*, 1980). In addition, two interesting reviews on sialidases from microorganisms and mammals (Corfield *et al.*, 1981a; Veh & Sander, 1981) have been included, together with other reports on sialidases and sialidoses (see Tettamanti *et al.*, 1981). Furthermore, other papers on neuronal sialidases have been published in the book edited by Gatt *et al.* (1978), and others on sialidases and sialidoses in the book edited by Svennerholm *et al.* (1980).

Although glycosidases from some marine gastropods (see Egami, 1974) and from the terrestrial snail *Helix pomatia* (Marnay *et al.*, 1964) were studied between approximately 1957 and 1974, few attention has been paid to the *glycosidases from other molluscs*.

Our studies mainly concern several glycosidases characterized from some molluses and vertebrates, not previously investigated, and with neuraminidase purified from microbial sources.

The present paper is a review on glycosidases from animal sources (except for neuraminidase), related with the results that appeared after 1978, taking into consideration that many topics on glycosidases have been summarized in the above mentioned reviews.

3. α-L-FUCOSIDASE (EC 3.2.1.51)

3.1 *α-L-Fucosidase from some marine molluses*

Reglero & Cabezas (1976) have obtained a pure preparation of α-L-fucosidase from the liver (hepatopancreas) of the marine mollusc *Chamelea gallina* L. (old name, *Venus gallina* L.). It not only hydrolyzes *p*-nitrophenyl α-L-fucoside but also natural substrates such as oligosaccharides containing fucosidic residues with α1 2, α1 3 and α1 4 linkages, fucose-containing glycopeptides such as thyroglobulin glycopeptide, and glycoproteins as porcine submaxillary mucin (previously rendered free of sialic acid).

These results explain that it may be classified under

Table 1. Characteristics of α-L-fucosidase from the hepatopancreas of several marine molluscs

Properties	*Ch. gallina*	*T. rhomboideus*	*L. littorea*	*M. edulis*
Purification-fold	300	450	225	
pH optimum	5.0	5.0	6.5, 3.0	4.0
pI	6.3, 6.6	6.2	5.4	
K_m (mM)	0.07	0.14	0.3	0.12
V_{max} (μM·mg^{-1} per min)	3.5	4.1	2.6	0.4
Mol wt (k dalton)	200	200	220	–

the number EC 3.2.1.51 (Dixon & Webb, 1979) and not as under EC 3.2.1.63, corresponding to 1,2-α-L-fucosidase (IUB, 1979).

Some properties of this enzyme are very similar to those of the α-L-fucosidase purified (Cabezas & Reglero, 1977) from another marine mollusc, *Tapes rhomboideus* Penn. (see Table 1).

The α-L-fucosidase from the hepatopancreas of the marine mollusc *Littorina littorea* L. has also been studied (De Pedro *et al.*, 1978; Cabezas *et al.*, 1979, 1981); and that from *Mytilus edulis* L. (Cabezas *et al.*, 1982b). Their characteristics are summarized in Table 1. The enzymes from the three earlier sources are fully active after heating at 60 C for 5 min at pH values 5.0 5.5, but at 70 C the activity decreases very quickly.

Purification and properties of α-L-fucosidase from *Venus mercenaria* have been reported by Concha-Slebe *et al.* (1979).

3.2. *α-L-Fucosidase from blood of various mammalian species*

3.2. (a) *From human serum of patients with several disorders.* We have carried out studies on some glycosidase activities (α-L-fucosidase, β-*N*-acetylglucosaminidase, β-D-galactosidase, etc) in blood serum from patients diagnosed as having diabetes mellitus, hepatic cirrhosis, gastric carcinoma (Reglero *et al.*, 1980a) and renal deficiencies (Reglero *et al.*, 1980b). These studies were followed (Calvo *et al.*, 1981) by another similar work made on sera from patients of viral hepatitis, pancreatitis, myocardial infarction and breast cancer. Another paper related to β-*N*-acetylglucosaminidase and α-L-fucosidase in diabetics with retinopathy has just appeared (Miralles *et al.*, 1982). Table 2 summarizes some of the most relevant results.

Depending on the patient groups, there is a wide range of differences for the enzyme activities that we have determined. The highest differences between normal controls and patients sera are generally for α-L-fucosidase and for β-*N*-acetylglucosaminidase activities in diabetes mellitus, hepatic cirrhosis and gastric carcinoma (Reglero *et al.*, 1980a).

Obviously the differences in activities that we found between the assayed glycosidases from human groups could be related to the concentration and intrinsic activity of each enzyme towards its respective *p*-nitrophenyl derivative used as substrate. However, for pathological sera perhaps the differences in comparison with controls are due to the different specificities for catabolic recognition and clearance of these glycosidases: furthermore, the original organ specificity corresponding to the enzymes which are circulating in blood could probably play a role in the half-life of each enzyme.

The determination of some of these activities in sera from patients who ingested a toxic agent also shows differences in some activities with respect to controls (Cabezas-Delamare *et al.*, submitted for publication).

Wood (1976) reported that an extremely low activity may be observed when α-L-fucosidase activity is determined in blood from some normal individuals. Bernard *et al.* (1979, 1981) have studied this fact and have characterized two major forms of the enzyme which could perhaps explain the differences in the activity due to their variable proportions. In addition, Willems *et al.* (1982) have confirmed the existence of a plasma α-L-fucosidase polymorphism and the autosomal recessive inheritance of the low activity trait.

Other papers concerning to α-L-fucosidase from human placenta (Turner, 1979), human liver (Thorpe & Oates, 1978), serum of hyperthyroid patients (Guillou *et al.*, 1982) and specificities of human glycosidases (Ben-Yoseph *et al.*, 1979) have been published.

3.2. (b) *From serum and leukocytes of various mammalian species.* In the previous section we have described the different activities which show several glycosidases of the human blood sera either in normal or in pathological samples. For example, *N*-acetylglucosaminidase activity is the highest (Reglero *et al.*, 1980a, 1980b; Calvo *et al.*, 1981), followed by α-L-fucosidase activity, and then by other glycosidases.

Another experimental approach is the determination of the same enzyme in blood sera of different animal species. The problem in this approach is the activity depending on the species specificity.

We have determined (Villar *et al.*, 1978) the α-L-fucosidase activity in sera from several animals in comparison with that from healthy human donors. The results, represented as percentage of the activity in comparison with the highest (100%), are as follows:

Human (100%) > pig (42.8%) > bull, Morucha race (6.8%) > horse (5.5%) > donkey (4.3%) > bull, Charolais race (3.4%) > goat (2.2%); these values are determined by the fluorimetric procedure using 4-methylumbelliferyl-α-4-fucopyranoside as substrate, and parallel results (but lower values) in the spectrophotometric assay, using *p*-nitrophenyl α-fucoside as substrate. Thus, α-L-fucosidase activity shows big differences for the same material (blood serum) in some species and even for two races belonging to the same species (Morucha and Charolais bull).

In addition, α-L-fucosidase of pig and mule leukocytes from circulating blood exhibits the same pH optima (4.0), but the latter has double specific activity and higher thermal stability in comparison with these

of pig with *p*-nitrophenyl-α-fucoside as substrate (Sevillano *et al.*, 1978).

Recently, Watkins & Alhadeff (1981) have reported differences in liver α-L-fucosidase from vertebrates belonging to several species (human, macaque, marmoset, rat, camel, zebra, dingo, armadillo, boa and fish) with respect to pH optima, thermostabilities and pI, but similar K_m. Studies on molecular forms of monkey brain α-L-fucosidase have been carried out by Alam & Balasubramanian (1978, 1979). On the other hand, Alhadeff & Andrews-Smith (1980) have found that neuraminidase treatment of human liver α-L-fucosidase does not change its thermostability.

4. β-D-FUCOSIDASE (EC 3.2.1.38)

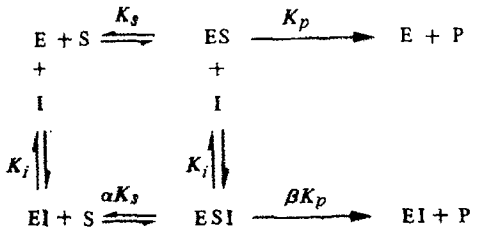
4.1. *β-D-Fucosidase, β-D-glucosidase and β-D-galactosidase activities associated in molluscs*

4.1 (a) *In the mollusc* Helicella ericetorum *Müll.* In the first official report on Enzymes of the International Union of Biochemistry (IUB, 1961) β-fucosidase was not present and β-galactosidase and β-glucosidase were classified as independent enzymes; β-fucosidase was considered to be a side activity of β-galactosidase. β-Fucosidase was included as an independent enzyme in 1964 (IUB, 1965) and, unexpectedely, deleted in 1972 (IUB, 1973). Finally, it has been reclassified in the last official Recommendations on Enzyme Nomenclature (IUB, 1979).

Previous results of our Department (Calvo *et al.*, 1979) have suggested the occurrence of β-D-fucosidase with different characteristics than β-D-galactosidase in several materials.

The digestive gland of the terrestrial snail *H. ericetorum* is a rich source of a glycosidase (Calvo *et al.*, 1978a; 1980) that possesses β-D-fucosidase, β-D-glucosidase and β-D-galactosidase activities, all associated in a single peak in both DEAE cellulose chromatography and isoelectric focusing (pI 4.35), having the same optimal pH (5.0) (Calvo *et al.*, 1982). β-D-fucosidase shows the lowest K_m (0.37 mM), the highest V_{max} and the best V_{max}/K_m value (Calvo *et al.*, 1979; Cabezas *et al.*, 1982b) with the corresponding *p*-nitrophenyl glycosides as substrates. Close activity values are obtained for β-D-glucosidase, but much lower activity is found for β-D-galactosidase (Cabezas *et al.*, 1982b).

Furthermore, β-D-fucosides and β-D-glucosides compete completely for a common active site in mixed-substrate experiments, while β-D-galactosides only partially compete with both glycosides. With δ-D-gluconolactone, the enzyme shows a hyperbolic mixed-type inhibition (Segel, 1975) mainly competitive for β-D-fucosidase and β-D-glucosidase activity (with the same inhibition subtype), and predominantly non-competitive for β-D-galactosidase activity (with different inhibition subtype) according to the following scheme:

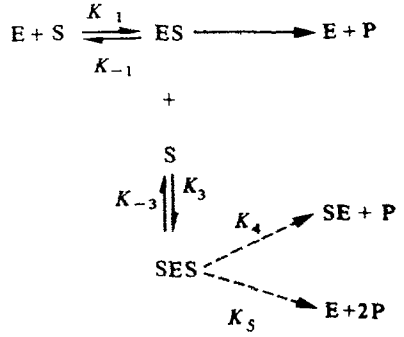


The Dixon plots (Dixon, 1953) are not linear, but convex upward for all activities (Calvo *et al.*, 1982).

All the kinetic evidences suggest that this enzyme has two active sites, a *fuco-gluco* site and a *galacto* site. This result is similar to that found by Conchie *et al.* (1967) in the limpet β-D-galactosidase, explained for the fitting of the substrate in the active site (see Fig. 1).

The activation of the enzyme by some carbohydrates (Calvo *et al.*, 1982) could be explained by a mechanism of transglycosylation assigned to these enzymes (Wallenfels & Malhotra, 1961).

4.1. (b) *In the mollusc* Littorina littorea *L.* We have found (Melgar *et al.*, 1982) that the digestive gland of the periwinke, *L. littorea*, is a very rich source of a glycosidase that possesses β-D-fucosidase, β-D-glucosidase and β-D-galactosidase activities associated in a single peak in Sephadex G-200, DEAE cellulose and affinity (agarose-aminocaproyl-β-D-galactosylamine) chromatographies, and in isoelectric focusing (pI 4.25), having the same optimal pH (5.5). These results are similar to the above-mentioned results obtained in *H. ericetorum*. However, the enzymes of both sources differ by some kinetic peculiarities. Thus, different K_m and V_{max} values were found (Cabezas *et al.*, 1982b) when using low (0.05–0.2 mM) and high (1.5–4 mM) substrate concentrations in the assays with the enzyme of *L. littorea*. This suggests a substrate-activation model, similar to that proposed by Frieden (1964). At high substrate concentration the enzyme–substrate complex would bind a second substrate molecule, and the resulting SES complex would yield product at a higher velocity. The second substrate molecule can behave as an activator modifier (SES → SE + P) or as a substrate (SES → E + 2P). In summary:



The secondary binding site would be the alternate active site (the *galacto* site for β-D-fucosidase and β-D-glucosidase activities and the *fuco-gluco* site for β-D-galactosidase activity).

In addition, when mixed-substrate analysis is carried out (Melgar *et al.*, 1982), β-D-fucosides and β-D-glucosides compete completely for a common active site while β-D-galactosides only partially compete with either β-D-fucosides or β-D-glucosides. This suggests that β-D-fucosidase and β-D-glucosidase activities are catalyzed in the same active site, while β-D-galactosidase activity is mainly catalyzed in a different active site.

On the other hand, the coincident K_i values for β-D-fucosidase and β-D-glucosidase activities, with D-fucose, glucose and galactose and the very different

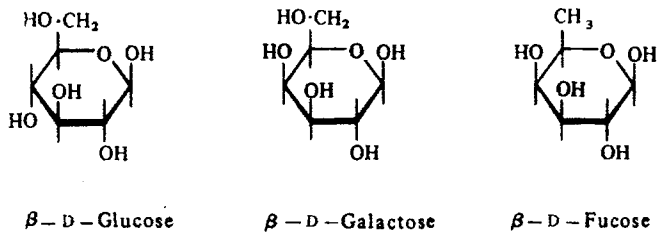
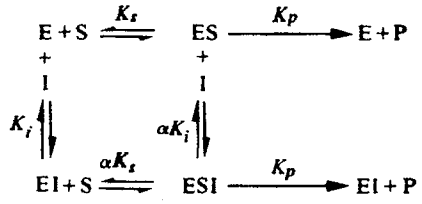


Fig. 1. Carbohydrate moiety of some substrates of the β-D-fucosidase.

K_i values for β-D-galactosidase activity with those inhibitors (Cabezas *et al.*, 1982b) are consistent with the two-site models proposed for this enzyme. The enzyme exhibits with the above-mentioned sugars a partial competitive-type inhibition following this scheme:



Furthermore, the Dixon plots (Dixon, 1953) are also not linear, but convex upward. Thus, all the kinetic evidence suggest that this enzyme has two different active sites: a *fuco-gluco* site and a *galacto* site.

On the other hand, at high substrate concentrations, some activities are activated by D-fucose, glucose and galactose, probably in relation with a transglycosylation mechanism.

4.2. *β-D-Fucosidase, β-D-glucosidase and β-D-galactosidase activities associated in mammals*

4.2. (a) *In rabbit liver.* The enzyme with β-galactosidase and β-glucosidase activities which we have studied (Llanillo *et al.*, 1977) in rabbit liver hydrolyzes *p*-nitrophenyl β-D-glucoside, *p*-nitrophenyl β-D-galactoside and *p*-nitrophenyl β-D-fucoside at a ratio of 1:0.6:0.4, which remains constant throughout the whole purification procedure.

β-D-Glucosidase and β-D-galactosidase activities show similar profiles by chromatography through Sephadex G-200 and DEAE-A50 Sephadex. The thermal stabilities, pH optimum and electrophoretic mobilities of the protein corresponding to both activities are also coincidental. That and other evidence suggest that these two enzyme activities reside in the same protein molecule. The possibility that an independent β-D-fucosidase activity (single activity) was responsible for the hydrolysis of β-D-fucoside substrate has been discarded.

The inhibition studies demonstrate that the enzyme has a single active site responsible for the hydrolysis of three substrates, since in every case the rate of hydrolysis of the mixed substrates is less than the sum of the rate obtained with separate substrates.

These properties found in our Department have been taken into consideration by the IUB Committee (1980) to recommend the addition of the following paragraph to the comments of the β-D-galactosidase (IUB, 1979): "some animal enzymes also hydrolyze β-D-fucosides and β-D-glucosides".

4.2. (b) *In bovine liver.* β-D-Fucosidase, β-D-glucosidase and β-D-galactosidase activities are also associated in bovine liver, in a single peak in isoelectric focusing (pI 4.35) for all these activities, suggesting that they are catalyzed by the same enzyme (Rodríguez *et al.*, 1982; Calvo *et al.*, 1982).

This enzyme shows the optimal pH in the range 4.5–6.5 for the above-mentioned activities. It shows the highest V_{max} with *p*-nitrophenyl β-D-fucoside as substrate and the lowest K_m with *p*-nitrophenyl β-D-glucoside. According to the V_{max}/K_m (Sols & Crane, 1954), the glucoside derivative is the best substrate, and the galactoside derivative the worst one.

The Lineweaver–Burk profiles (Lineweaver & Burk, 1934) are convex upward in most cases, suggesting a substrate-activation model, as discussed above, and the presence of more than one binding site in the enzyme.

D-Fucose is the strongest inhibitor, in comparison with glucose and galactose, the inhibition being competitive in all cases.

4.3. *Comments*

From the first report on β-D-fucosidase activity (Levvy & McAllan, 1963a) and its relation to β-D-galactosidase (Levvy & McAllan, 1963b) carried out in molluscs and in plant seeds (Cabezas & Vazquez Pernas, 1969), several years were spent without remarkable progress on this topic.

Later, other authors studied this enzyme in pig kidney (Wiederschain & Prokopenkov, 1973) and human kidney (Wiederschain *et al.*, 1981). Recently, Daniels *et al.* (1981) found a cytosolic β-glucosidase in human liver that hydrolyzed the 4-methylumbelliferyl derivatives of β-D-galactose, β-D-fucose, β-D-xylose and α-L-arabinose, as well as steroid-glucosides and arylgalactosides.

On the other hand, Norden *et al.* (1974) have purified a G_{M1}-ganglioside β-galactosidase A (EC 3.2.1.23) from human liver than releases D-galactose from synthetic (chromogenic) β-D-galactosides as well as natural substrates. This enzyme also hydrolyzes β-D-fucoside and α-L-arabinoside linkages. Recently, Wiederschain *et al.* (1981) studied the hydrolysis of fucosides, galactosides and arabinosides by partially purified enzyme from human kidney and found that the assayed glycosides are hydrolyzed by the same type of fucosidase: β-D-fucoside (β-D-galactoside, α-L-arabinoside) hydrolase. They confirm that data on the specificity of different types of fucosidase may be useful in the differential diagnosis of some glycosidoses.

β-D-Fucosidase activity has also been found in other human material: in meconium from infants with cystic fibrosis (Antonowicz *et al.*, 1978).

Colas (1980) has followed his previous research on β-fucosidase from a giant African snail, *Achatina balteata*, showing that the catalytic efficiency is maximum towards β-D-fucosides in comparison with β-D-glucosides and β-D-galactosides. He has proposed the presence in the enzyme molecule of at least two distinct sites for the substrates, one being an active site and the other one being either a second active site with different kinetic parameters or a modifier site.

In summary: (a) from our own results and those of other authors, obtained in different materials, it seems that the occurrence of β-D-fucosidase as a well characterized enzyme activity is definitely established.

(b) There is a wide variability in the properties of β-D-fucosidase concerning the association to other activities (β-D-galactosidase, β-D-glucosidase, α-L-arabinosidase). The structural similarities between the sugar moieties of the respective *p*-nitrophenyl derivatives employed as substrates in these studies is probably related with this fact.

5. β-D-GALACTOSIDASE (EC 3.2.1.23), α-D-MANNOSIDASE (EC 3.2.1.24), α-D-GALACTOSIDASE (EC 3.2.1.22), β-D-GLUCOSIDASE (EC 3.2.1.21) AND α-D-GLUCOSIDASE (EC 3.2.1.20)

5.1. *β-D-Galactosidase from rabbit spleen: separation of two forms*

Although β-D-galactosidase, an enzyme widely distributed in mammalian tissues, consists of series of molecular forms, the number and relationship between these forms have not been well defined. Rabbit spleen, a source not previously investigated, contains a β-D-galactosidase which shows heterogeneity by isoelectric focusing. Two forms, **I** and **II**, are separated by DEAE cellulose chromatography. They exhibit a different heat stability as well as a different pI (6.7 for form **I**, and 5.3 and 6.7 for form **II**). In addition, form **I** is resolved in a single peak of mol wt 75,000 by gel-filtration, whereas form **II** is eluted in one or two peaks, depending on the pH elution, of mol wt 120,000 and >200,000. However, other characteristics (optimal pH, K_m, behaviour against inhibitors) with *p*-nitrophenyl β-D-galactoside as substrate are similar for both forms (Rodriguez-Berrocal *et al.*, 1981; Rodriguez-Berrocal, 1982).

Two forms of β-galactosidase from newborn rat epidermis have been separated by DEAE cellulose chromatography (Miyagawa, 1979), and also from feline liver (Holmes & O'Brien, 1979). The occurrence of multiple forms of β-galactosidase in human liver has been previously reported and the stability and aggregation properties have been studied by Heyworth *et al.* (1981).

5.2. *β-D-Galactosidase, α-D-galactosidase, α-D-mannosidase, β-D-glucosidase in human pathological sera, and in sera from several animal species*

Table 2 shows that *β-D-galactosidase* activity is significantly increased in diabetes mellitus, hepatic cirrhosis and gastric carcinoma, but it decreases in the other disorders studied by us.

Higher values of *α-D-galactosidase* activity are found in comparison with controls in gastric carcinoma ($P < 0.01$) and hepatic cirrhosis ($P < 0.05$). However, not significant differences in neither diabetes mellitus (Reglero *et al.*, 1980a) nor renal disturbances (Reglero *et al.*, 1980b) are detected.

Schram & Tager (1981) have recently outlined that the original concept of the α-galactosidase occurring in various tissues and body fluids from humans requires modification, since α-galactosidase **B** should be referred to as *N*-acetylgalactosaminidase. Furthermore, the use of artificial substrates can lead to confusion in this and other cases, according to these authors.

No significant differences are found for *α-D-mannosidase* in all the above-mentioned disorders (Reglero *et al.*, 1980a, 1980b).

β-D-Glucosidase shows increased activity in sera from patients with acute viral hepatitis ($P < 0.001$), acute pancreatitis ($P < 0.001$), breast cancer ($P < 0.001$) and acute myocardial infarction ($P < 0.05$) (Calvo *et al.*, 1981).

α-D-Glucosidase exhibits a parallel pattern with β-D-glucosidase, but with lower differences between pathological and normal sera. Thus, the differences are, for viral hepatitis, $P < 0.001$; for acute pancreatitis, $P < 0.1$; for breast cancer, $P < 0.1$, and for acute myocardial infarction, $P < 0.05$ (Calvo *et al.*, 1981).

The signification of other enzymes (α-L-fucosidase and β-D-glucuronidase) in these disorders has been discussed before and that of β-*N*-acetylhexosaminidase will be studied later.

All above-mentioned activities of this section have also been determined in sera from *Bos taurus* L. (bull), *Capra hircus* L. (goat) and *Sus scropha* var *domestica* L. (pig) (García *et al.*, 1979) using both fluorimetric and spectrophotometric procedures. Generally, these activities are the highest for goat, followed by pig and bull, with respect to the corresponding activity of

Table 2. Relative glycosidase activities in normal and pathological sera

Enzyme	Diabetes mellitus	Hepatic cirrhosis	Gastric carcinoma	Breast cancer	Acute myocardial infarction	Pancreatitis	Renal deficiencies
α-L-Fucosidase	504	577	699	105	114	123	275
β-*N*-Acetylglucosaminidase	118	164	120	136	133	173	68
β-D-Galactosidase	126	144	157	89	61	88	90

Relative activity of mean values (of 10 patients each group) in comparison with a 100% assigned to the control group (of 10 normal subjects).

human source. Radin (1978) has studied the inhibitors of β-glucosidase of animal tissues.

6. β-*N*-ACETYLHEXOSAMINIDASE (EC 3.2.1.52)

The occurrence of a high β-*N*-acetylhexosaminidase (also designed as hexosaminidase) activity in very different materials (hepatopancreas from molluscs, mammalian tissues and sera, etc) is one of the common properties of this enzyme. In some cases this high activity constitutes an additional difficulty in the purification of other glycosidases also present in the original source, frequently with a lower activity.

Another peculiarity of the β-*N*-acetylhexosaminidase is the occurrence of two activities, β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase, catalyzed by the same enzyme. Although one of these activities, β-*N*-acetylglucosaminidase, is generally the predominant, the other one remains in the most pure preparations. Furthermore, the ratio of both activities is the same in all steps of the purification, at least in the materials that we have studied (see later).

Furthermore, the occurrence of several forms in many β-*N*-acetylhexosaminidases is another property investigated in this enzyme, as it is in other glycosidases. However, differences in thermal stability, etc, have been observed in several forms of the β-*N*-acetylhexosaminidase depending on the source.

6.1. β-N-*Acetylhexosaminidase from molluscs*

We have studied the β-*N*-acetylhexosaminidase from hepatopancreas of the marine mollusc *Chamelea gallina* L. (=old name, *Venus gallina* L.) (Pérez & Cabezas, 1977), and that of the terrestrial molluscs *Helicella ericetorum* Müll. (Calvo *et al.*, 1978a) and *Arion rufus* L. (Villar *et al.*, 1979; Villar, 1982).

The ratio for β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities remains constant during all steps of the purification procedure, being 3:1, 4:1 and 3.5:1, respectively, for the enzyme of the three above-mentioned sources.

We have observed a very different behaviour in the thermal stability of this enzyme. Thus, β-*N*-acetylhexosaminidase from *Ch. gallina* loses 97% of its activity after incubation at 60°C for 5 min. In contrast, that of *H. ericetorum* only loses 10% of its activity at the same-incubation conditions, and β-*N*-acetylhexosaminidase from *A. rufus* contains two forms, one which shows thermal stability and another which exhibits thermal lability.

Mixed-substrates analysis and K_i values for competitive inhibitors (Cabezas *et al.*, 1982b) indicate that β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities of the β-*N*-acetylhexosaminidase purified from *H. ericetorum* are catalyzed at the same active site. Furthermore, two forms of the enzyme are separated by preparative polyacrylamide-gel electrophoresis. In addition, this enzyme acts as an endoglycosidase towards natural substrates (ovalbumin, ovomucoid, chondroitin 4-sulphate, chitin and hyaluronic acid), and it is also very active against the respective *p*-nitrophenyl derivatives (Cabezas *et al.*, 1981b). Thus, it shows several characteristics very different from other β-*N*-acetylhexosaminidase (Calvo *et al.*, 1978b).

The effect of immobilization with bovine serum albumin and glutaraldehyde on stability and properties of β-*N*-acetylhexosaminidase from the marine mollusc *Turbo cornutus*, as well as the purification and characterization of two forms from this source, have been studied by other authors (Yeung *et al.*, 1979a,b). Santoro & Dain (1981) have made a comparative study of β-*N*-acetylglucosaminidase from three clams species: *Mercenaria mercenaria* (quahog); *Mya arenaria* (shoft-shelled clam) and *Spisula solidissima* (surf clam). An increase with raised salinity in the β-*N*-acetylhexosaminidase activity of *Mytilus edulis* L. has been reported (Moore *et al.*, 1980; Bayne *et al.*, 1981).

Except for all these molluscs species, it seems that others have been scarcely investigated in what concerns to β-*N*-acetylhexosaminidase.

6.2. *Acetylhexosaminidases from normal vertebrate tissues and sera*

The presence of different forms of β-*N*-acetylhexosaminidase in several mammalian tissues is known after 1968 (see Reglero *et al.*, 1981a). The occurrence of three forms (A, B and I) in horse brain and their characterization has been reported (Reglero *et al.*, 1981a).

Overdijk *et al.* (1981b) have also characterized a third form of enzyme, hexosaminidase C, from bovine brain. The structural difference between the β-chains in hexosaminidase A and B from purified placenta has been described (Mahuran & Lowden, 1981). Other studies have been published on hexosaminidases A and B from the human placenta (Geiger & Arnon, 1978; Freeze *et al.*, 1979).

Furthermore, two enzyme forms, A and B, have been separated from *Bos taurus* (bull) and *Capra hircus* L. (goat) sera by DEAE-cellulose chromatography and three forms, A, B and S, from *Sus scropha* L. (pig) serum, the B forms being the most heat-stable and the A forms the least stable (Calvo *et al.*, 1978).

On the other hand, isoelectric focusing revealed no differences in primates (Lee *et al.*, 1979).

The unusual molecular weight forms of β-*N*-acetylhexosaminidase from intestinal mucosa of newborn pigs (Munn *et al.*, 1979) and the changes in its specific activity in the rat epididymis with age (Milne *et al.*, 1978) have also been studied.

Sarber *et al.* (1978) have investigated the β-*N*-acetylhexosaminidase from bovine testes. The occurrence of several forms of β-*N*-acetylhexosaminidase is not exclusive of mammalian tissues. A "neutral" hexosaminidase has been separated from other hexosaminidase forms (I and II) and characterized in embryonic and 1-day old chicken brains in our laboratory (Sanchez-Bernal *et al.*, 1982).

Another area in the wide field of research on hexosaminidase is that of its activity toward substrates and inhibitors. The hydrolysis of various oligosaccharides by β-*N*-acetylhexosaminidases A and B isolated from human liver has been reported by Spik *et al.* (1979), and that of *N*-acetylglucosamine-6-sulphate by human placenta and skin fibroblasts β-*N*-acetylhexosaminidase A (Kresse *et al.*, 1981). We have found that β-*N*-acetylglucosaminidase A and B from porcine placenta, bovine epididymis and horse brain is inhibited

by mannose and other carbohydrates (Reglero *et al.*, 1979; Reglero, 1979).

On the other hand, the demonstration of an endo-β-*N*-acetylglucosaminidase activity in human tissues has been reported (Overdijk *et al.*, 1981a), by Tarentino & Maley in hen oviduct (1978), and by Delmotte *et al.* (1979) in rabbit serum. Furthermore, the cytosolic location of an endo-β-*N*-acetylglucosaminidase in rat liver has also been described (Pierce *et al.*, 1979, 1980).

Studies on placenta α-*N*-acetylglucosaminidase (Röhrborn & von Figura, 1978), endo-α-*N*-acetylgalactosaminidase (Umemoto *et al.*, 1978) and α-*N*-acetylgalactosaminidase (Salvayre *et al.*, 1979, 1981) have also been carried out.

6.3. β-N-*Acetylhexosaminidase from pathological mammalian tissues, sera, urine and other materials*

The properties of β-*N*-acetylhexosaminidase from human fibroblasts (Miller *et al.*, 1981) and their pinocytosis (Kaplan, 1980) have also been investigated. And the compartmental distribution of β-hexosaminidase isozymes in I-cell fibroblasts has been described (Vladutiu & Rattazzi, 1981).

Although the metabolism of ethanol by oxidising systems has been studied in detail by several authors, its effects on lysosomal enzymes has not been practically investigated. We have determined β-*N*-acetylglucosaminidase and β-D-glucuronidase, as lysosomal activities, as well as other enzyme activities from other subcellular localizations, in livers and brains from alcoholic rats (García *et al.*, 1980). Generally, the difference between problems and controls from brain fractions are less than in the respective liver fractions. In the lysosomal enriched fractions β-*N*-acetylhexosaminidase activity is always higher in control than in ethanol-intoxicated rats. A protective effect of the ethanol towards the lysosome membrane could perhaps explain this result.

In streptozotocyn-diabetic mice we have observed (Serrano *et al.*, unpublished work; Calvo *et al.*, 1982) that there is not a significant difference compared with controls for β-*N*-acetylhexosaminidase (both β-*N*-acetylglucosaminidase and β-*N*-acetylgalactosaminidase activities) in homogenates from brain, liver, spleen and heart. In contrast, kidneys from diabetic mice show less activity (20%) for β-*N*-acetylglucosaminidase and higher activity (125%) for β-*N*-acetylgalactosaminidase. Generally, other glycosidase activities also exhibit significant differences (higher or lower) in comparison with controls in kidney homogenates, but not for the other above-mentioned organs.

The occurrence of multiple forms in β-*N*-acetylhexosaminidase from human serum of cancer patients (Lo & Kritchevsky, 1979), from colonic tumours of rats (Mian *et al.*, 1979a), from tissues and media from human colonic adenocarcinoma (Tsao *et al.*, 1979), as well as the presence of an atypical thermolabile species of β-hexosaminidase B in metastatic-tumour tissue of human liver (Alhadeff & Holzinger, 1982), has been described. Tucker *et al.* (1980) have also studied human β-*N*-acetylhexosaminidase isoenzymes as an indicator of tissue damage in disease.

The increased activity of β-*N*-acetylglucosaminidase in pregnancy serum has been investigated by several authors (Lowden, 1979; Hultberg & Isaksson, 1981), and also the influence of the age (Lombardo *et al.*, 1981) and its activity in several lysosomal disorders (Hultberg *et al.*, 1980).

Others results are indicated in Table 2. It shows that in sera from diabetes mellitus, hepatic cirrhosis, gastric carcinoma, heart cancer, acute myocardial infarction and pancreatitis patients, β-*N*-acetylglucosaminidase activity is significantly increased. In several deficiencies this activity is lower than in controls. These results have been previously discussed (Reglero *et al.*, 1980a, 1980b; Calvo *et al.*, 1981). Recently, we have also confirmed (Miralles *et al.*, 1982) that β-*N*-acetylhexosaminidase activity is higher in sera from diabetic patients, independent of the development of retinopathy and also independent of the length of diabetes.

On the other hand, it has been observed in our laboratory (Sanchez-Bernal *et al.*, 1983) that urine from rats which have received intraperitoneally the antibiotic gentamycin for several days show a higher β-*N*-acetylhexosaminidase activity than controls. However, 4 days after interruption of treatment the values become normal. Furthermore, β-galactosidase exhibits a similar pattern, but not other glycosidases.

Recently, Hirani *et al.* (1982) have characterized β-*N*-acetylhexosaminidase B from human urine.

The clinical significance of β-*N*-acetylhexosaminidase activity has also been studied in materials such as human tears (van Haeringen *et al.*, 1978) and human hair roots (Phillips & Thorpe, 1978). Its presence in human breast milk has also been reported (Overkotter *et al.*, 1982).

7. β-D-GLUCURONIDASE (EC 3.2.1.31)

7.1. β-D-*Glucuronidase in the molluscs* Littorina littorea *L. and* Helicella ericetorum *Müll*

Although the occurrence of multiple forms of this enzyme in organs of several mammalian species has been reported (see Diez & Cabezas, 1979), we had not found any reference to this multiplicity in marine molluscs. The occurrence of two molecular forms, **I** and **II**, in the β-glucuronidase of the liver (hepatopancreas) from the marine mollusc *Littorina littorea* L. has been demonstrated (Diez & Cabezas, 1979; Cabezas & Diez, 1979). Form **I** was purified about 355-fold and form **II** 1260-fold. They behaved differently on DEAE cellulose chromatography, polyacrylamide gel disc electrophoresis, isoelectric focusing, optimal pH, thermal stability and K_m with *p*-nitrophenyl- and phenolphthalein-glucuronide as substrates. This β-glucuronidase is a glycoprotein, in which sialic acid was not detected, its mol. wt is about 250,000, and several subunits were separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. This enzyme is very active on synthetic substrates and also on hexasaccharides and tetrasaccharides containing glucuronic acids with β1–3 linkages, but it has practically no activity on certain glycosaminoglycans (Cabezas *et al.*, 1981b).

In a similar manner, we have also found two enzyme forms possessing β-glucuronidase activity in the terrestrial mollusc *Helicella ericetorum* Müll. (Reglero *et al.*, 1981b). These forms were separated by

DEAE–cellulose chromatography and differ by their pI (6.0 and 5.0, respectively), thermal stability and K_m and V_{max} with *p*-nitrophenyl β-D-glucuronide as substrate.

Microsomal and lysosomal β-glucuronidase (see before, introduction) have been reported in rat liver. One may wonder if the occurrence of several forms in β-glucuronidase from hepatopancreas of *L. littorea* and *H. ericetorum* is also dependent to a different subcellular localization of the enzyme in this material. We have tried to check it, but a pure preparation of lysosomes from these molluscs was not achieved.

On the other hand, the occurrence of multiple forms of β-glucuronidase in mammalian tissues (Diez & Cabezas, 1979; Reglero *et al.*, 1981a) and in the above-mentioned mollusc species, has been enlarged by the demonstration of several forms in chick embryo liver (Glaser & Conrad, 1980).

7.2. *β-D-Glucuronidase in human pathological materials and in sera from diabetic mouse*

We have found a significantly increased β-D-glucuronidase activity in comparison with controls in blood sera from patients with hepatic cirrhosis, a lower difference for diabetes mellitus, and no difference in neither gastric carcinoma (Reglero *et al.*, 1980a) nor renal disturbances (Reglero *et al.*, 1980b).

However, according to Sulochana *et al.* (1982), the activity of β-glucuronidase in the gastric aspirate from cases of human carcinoma of the stomach is markedly elevated when compared with normals.

In addition, heart homogenates from mice with diabetes mellitus (induced by streptozotocin) show a significantly high β-glucuronidase activity (Serrano *et al.*, unpublished work) in comparison with controls.

The study of this enzyme could be of special interest, taking into consideration its ability to split glucuronides, substances which are formed by detoxication mechanisms. However, the interpretation of some results could be more complicated than in other cases since this enzyme is localized in several subcellular fractions, at least in some materials.

8. NEURAMINIDASE OR SIALIDASE (EC 3.2.1.18)

This enzyme, acylneuraminyl hydrolase, shows remarkable differences (in mol. wt, kinetics, etc) according to the various sources (orthomyxovirus, paramixovirus, bacteria, protozoa, mammals). For example, in viruses such as orthomyxovirus it is a protein independent of hemmaglutinin (see Cabezas, 1978). However, in paramyxovirus the same protein has neuraminidase and hemmaglutinin activities (but there are paramyxovirus, such as *Morbillivirus* and *Pneumovirus*, in which neuraminidase has not been detected). In bacteria such as *Vibrio cholerae* (*comma*), the neuraminidase is an exoenzyme. On the contrary, in virus and mammals it is associated to the membranes. Furthermore, there are different forms of the enzyme, at least in some mammals (and perhaps in microorganisms), with different cellular localization (lysosomes, Golgi, citoplasmic membrane, cytosol fractions) in the case of the mammals. All these peculiarities could perhaps explain the different characteristics and behaviour of this enzyme. In connection with these facts, it has been proposed to employ the name "neuraminidase" for the enzyme from procaryotics, and "sialidase" for that of eucaryotics, but it has not yet been generalized. This is our approach concerning the following areas.

8.1. *Comparative studies on some procedures of neuraminidase determination, and assays on inhibitors*

We have compared (Cabezas *et al.*, 1980; Cabezas *et al.*, 1982a, 1982b) the following procedures to measure the neuraminidase activity in samples of neuraminidase purified from a strain of virus influenza A (H3N2), identical to the prototype A/Hong Kong/68 (H3N2), and/or from the bacterial species (*Vibrio cholerae* (*comma*) and *Clostridum perfringens*):

(a) spectrophotometric determination, at 340 nm, of the NADH produced in the coupled reactions summarized as follows:

$$N\text{-Acetylneuraminyl-lactose} + H_2O \xrightarrow{\text{neuraminidase}} N\text{-acetylneuraminic acid} + \text{lactose}$$

$$\text{Lactose} + H_2O \xrightarrow{\beta\text{-galactosidase}} \beta\text{-galactose} + \text{glucose}$$

$$\beta\text{-Galactose} + NAD^+ \xrightarrow{\beta\text{-galactose dehydrogenase}} \text{galactonic acid} + NADH + H^+$$

(This procedure is commercialized by Boehringer–Mannheim).

(b) release of α-methoxyphenol by the enzyme activity from 5-*N*-acetyl-2-*O*-(3-methoxyphenyl-α-D-neuraminic acid). This methoxyphenol yields a coloured quinone with 4-aminoantipyrine in the presence of potassium ferricyanide, as described by Santer *et al.* (1978).

(c) periodate-thiobarbiturate methods of Warren (1959) or Aminoff (1961) for free sialic acids determination, released from several substrates.

(d) the fluorescence produced by the aglycon split from the fluorigenic substrate 2'-(4-methylumbelliferyl)-α-D-acetylneuraminic acid (Warner, 1979), measured with excitation at 365 nm and emission at 450 nm.

We have found that the determination of the neuraminidase activity by the first procedure (spectrophotometric measure of NADH at 340 nm) appears satisfactory, although its sensitivity is about half that of Santer *et al.* (1978) and a tenth of periodate-thiobarbiturate procedures. However, this sensitivity could be highly increased by measuring NADH by fluorimetry (Cabezas *et al.*, unpublished results) instead of by spectrophotometry. Furthermore, in the

NADH and in the Santer *et al.* (1978) procedures there is no difference due to the reactions with the different types of sialic acid which appears in the methods of Warren (1959) or Aminoff (1961). In addition, the NADH procedure avoids the interferences produced in the periodate-thiobarbiturate methods by several substances such as sucrose (usually employed in the gradients for fraction procedures), L-fucose, deoxyribose, some glycosides and other compounds (see Cabezas *et al.*, 1980), and it can be employed to detect the neuraminidase activity in electrophoresis techniques if coupled with tetrazolium salts to stain gels.

Procedure (d) is a very sensitive procedure, although the substrate possesses a limited stability. On the other hand, it should be kept in mind that this and the substrate used in procedure (b) are synthetic substrates, so, it is recommended to also check the neuraminidase activity using natural substrates if neuraminidase deficiency studies are the aim of the study, taking into consideration that there are glycosidases that exhibit very big differences in their hydrolysis rates towards natural and synthetic substrates.

Another topic studied is the influence of some chemical compounds, assayed as neuraminidase inhibitors by the reaction of peryodate-thiobarbiturate, which interfere with this reaction and are not true inhibitors. This interference can be avoided with little modifications in the general procedure (Cabezas, 1978). Moreover, it has been observed that some Hg^{2+} salts, cytosine arabinoside and 6-azauridine are true inhibitors for the neuraminidase from several sources (Cabezas, 1978).

8.2. *Isoelectric focusing as a step in purification and characterization of viral neuraminidase*

Isoelectric focusing has been employed for the characterization of the neuraminidase. For the same strain of influenza virus A, we have found that the pI profiles of the neuraminidase preparations are different, depending on the purification procedures employed, for example, using proteolitic enzymes such as bromelain or the detergent *N*-lauroylsarcosine (Cabezas *et al.*, 1981a; Cabezas *et al.*, 1982a). This heterogeneity could be due to the different procedures employed for isolation and purification of the enzyme. However, the possibility that several forms of the enzyme could occur in the same strain of influenza virus A is not excluded.

In addition, isoelectric focusing may also be used as a step in the purification of neuraminidases. If methoxyphenol procedure is followed for the enzyme activity determination, dialysis of sucrose (usually employed for gradients) may be avoided. Furthermore, in the purification of the neuraminidase there is, in our experimental conditions, only about 20% of activity loss, a percentage similar to that resulting in other procedures.

8.3. *Sialidase in animal platelets and in rat brain*

In our Department, M. Cabezas (1972) found, for the first time, sialidase activity in a homogenate obtained from platelets isolated from ovine, equine (horse, donkey and mule) and porcine blood. He also confirmed the occurrence of this activity in bovine platelets. The enzyme releases sialic acid from *N*-acetyneuraminyllactose and pig submaxillary mucin at a similar rate, and higher than from fetuin and endogenous glycoproteins.

The activity of the sialidase towards endogenous substrates has been less studied than in exogenous (natural or synthetic) substrates. However, Cruz & Gurd (1981) have recently found that the sialidase present in the rat brain hydrolyzed 50–70% of endogenous GT_1 and GD_{1a} gangliosides at all ages, but GD_{1b} ganglioside was scarcely hydrolyzed by the neuraminidase from young rats, this hydrolysis ability being increased during the rat development.

8.4. *Specificity of microbial neuraminidase towards several substrates*

Vibrio cholerae (*comma*) neuraminidase is more active towards pig spleen gangliosides than neuraminidase from *Clostridium perfringens* (Hueso, 1982).

The neuraminidase from the influenza A/NT60/68 (H3N2), identical to A/Hong Kong/68 (H3N2), after liberation from the virus by *N*-lauroylsarcosine (Cabezas *et al.*, 1982a) is able to release sialic acids from very different substrates (Table 3).

As shown in Table 3, the assayed neuraminidase exhibits a wide specificity on several substrates which differs by: (i) their mol. wt; (ii) the type of sialic acid; (iii) the linkage of the predominant sialic acid. However, there are differences in the efficiency of the hydrolysis rate. In addition, other authors (see reviews) have found peculiarities in the hydrolysis of substrates for neuraminidases depending on the viruses and even on the viruses strains. Corfield *et al.* (1981a) have discussed in their excellent review the influence of the linkage, the carboxyl group, the *N*-substitution, the C_7 C_9 side chain and the *O*-acetylation of the sialic acids which are liberated from the substrates by sialidases from several sources. Corfield *et al.* (1981b) have also reported that the hydrolysis of NeuAc-containing compounds is easier than that of NeuGl-derivatives. Mian *et al.* (1979b) have observed kinetic differences in the hydrolysis of sulphate derivatives compared with unsubstituted substrates. Furthermore, Gatt *et al.* (1981) have pursued their research on the influences of bile salts on the hydrolysis of gangliosides and other glycoconjugates. Markwell *et al.* (1981) have studied the ability of gangliosides such as GD_{1a}, GT_{1b} and GQ_{1b} to function as natural receptors for Sendai virus in host cells.

Taking into consideration that sialic acids have been found in many materials (Cabezas, 1982) it is possible that neuraminidase activity will be detected in new sources. However, the occurrence of sialic acids in plants has not yet been demonstrated (Cabezas & Feo, 1969; Cabezas, 1982). Thus, the existence of sialidase in this material does not seem to be probable.

9. CONCLUDING REMARKS

After reading the previous pages, we could deduce that our knowledge on glycosidases has remarkedly improved during the last decade. However, many questions arise. Thus:

—Which is the turnover of the glycoproteins which are generally the glycosidases?

Table 3. Release of sialic acid(s) by neuraminidase purified from influenza virus A/NT 60/68 and by intact parainfluenza virus [ML 66, MT 56 and parainfluenza A (Sendai)]

Enzyme	Substrate	Type of the predominant sialic acid	Linkage of the predominant sialic acid, α	Hydrolysis rate (yield)
Purified neuraminidase from virus influenza A NT60/68	*N*-acetylneuraminyllactose	Neu5Ac	2–3	+ + + +*
	Fetuin	Neu5Ac	2 3	+ + + +
	Glycophorin A	Neu5Ac	2-6	+
	Bovine submaxillary mucin	Neu5Ac+ Neu5Gl+ Neu5AcylxAc	2-6	+ +
	Porcine submaxillary mucin	Neu5Gl+ Neu5Ac	2-6	+ +
	Equine submaxillary	$Neu4.5Ac_2$		±
	Gangliosides (from horse brain): a mixture of GD_{1a} (50%) + GM_1 (21%) + GD_{1b} (15%) + GT_{1b} (14%)	Neu5Ac	Prob. 2-3	+ + +
	Colominic acid	Neu5Ac	2-8	+
	4-Methylumbelliferyl-NeuAc	Neu5Ac	2–	+ + +
Purified virus parainfluenza (intact) from the following strains:	*N*-acetylneuraminyllactose	Neu5Ac	2-3	+ + +**
ML 66; MT 66; parainfluenza 1 (Sendai)	Fetuin	Neu5Ac	2-3	+ + + +
	α_1-Acid glycoprotein	Neu5AcxAc+ Neu5Ac	2-6 > 2-3	+ +
(Assayed independently)	Gangliosides (from pig lymphocytes): a mixture of $GM_3 > GD_3 > GD_{1a} > GM_1$	Neu5Gl		0

* These symbols are only used for comparative purposes, taking into consideration that the experimental conditions were not always equivalent with the substrates indicated.

** These symbols are valid only for comparative purposes when parainfluenza viruses (not purified neuraminidase) were employed. Thus, the results are not extrapolable to the assays with neuraminidase.

—What about enzyme replacement therapy?

—Will the biochemical engineering be able to produce synthetic glycosidases in a short time?

—What about the requirement of activator(s) for some enzymic hydrolysis of gangliosides?

We have not yet obtained a satisfactory answer for these and other questions. But we can expect that a success will reward the faith in this attempt.

9.1. *The metabolism of lysosomal enzymes* has been recently reviewed by Hasilik (1980). It is known that modifications corresponding to the protein and the carbohydrate moieties of the molecule occur during the processing of these enzymes. Other authors (Brunngraber, 1979; Montreuil, 1981) have also summarized interesting ideas on this topic.

From the basic research of Ashwell & Morell (1974) on the recognition phenomena and turnover of circulating glycoproteins, a big body of knowledge is emerging. Thus, the characterization of a liver receptor, which is also a glycoprotein, for rabbit liver (Ashwell & Morell, 1977), as glycoproteins are generally glycosidases and many natural substrates on which they act.

Stahl & Schlesinger (1980) have summarized the five sugar-specific pathways for the recognition of glycoconjugates, corresponding to phenomena in which are implicated the following carbohydrates or derivatives: galactose, mannose/*N*-acetylglucosamine, α-1,3-L-fucose, phosphomannose or acetylglucosamine. These authors postulate that a single receptor is able to participate in many uptake events, since it could be recycled. The role of mannose and mannose 6-phosphate as recognition markers is one of the most relevant features of this area, as indicated by Distler *et al.* (1979) for β-D-galactosidase, Goldberg & Kornfeld (1981) for β-glucuronidase, and Robey & Neufeld (1982) for β-hexosaminidase. Also Steer *et al.* (1979) and Kusiak *et al.* (1980) have studied the uptake of β-hexosaminidase, and Skudlarek & Swank (1981), the turnover of β-glucuronidase. In addition, microtubuli could play an important role in the lysosomal degradation of endogenous and exogenous proteins in hepatocytes (Grinde & Seglen, 1981).

On the other hand, a substantial decrease, age-related, in β-D-galactosidase activity of human liver cell lines seems to be due to either a slower synthesis or a more rapid proteolysis of the enzyme (Leray *et al.*, 1981). The turnover of β-galactosidase in fibroblasts from patients with genetically different types of β-galactosidase deficiency has been reported by Van Diggelen *et al.* (1981).

Other topics such as the regulation of glycosidases are scarcely understood.

9.2. About the *replacement therapy*, the review of Dawson (1978) has summarized the results obtained to that date. Other studies have been reported later by Rattazzi *et al.* (1980, 1981). It seems that some problems should be resolved before using this promising technique (see Tager *et al.*, 1974).

9.3. The *synthesis of some glycosidases by biochemical engineering* is theoretically possible, since other similar molecules have been obtained. However, the incorporation of the carbohydrate moiety of the glycoprotein molecule is an additional difficulty which should be kept in mind.

9.4. The occurrence of *protein activators for the hydrolysis of complex glycolipids* by lysosomal hydrolases could explain that a crude β-*N*-acetylhexosaminidase A, in the presence of this factor, acts on glycosphingolipids better than a pure preparation of this enzyme in the absence of the factor (Li & Li, 1981). Sandhoff & Conzelmann (1979) and Conzelmann *et al.* (1982) have recently reported the degradation of ganglioside GM_2 by hexosaminidase A depending on a specific activator protein. Also Li & Li (1982) have studied this topic, and Radin & Berent (1982) for β-glucosides. Let us hope than an approach to these and other not yet resolved problems will be achieved in a next future.

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When this paper was in press, the news of Professor Fujio Egami's death reached us. Let us dedicate this posthumous homage to him.

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